PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
MANDRAGOURAS, Amy, E.
Lahive & Cockfield, LLP
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ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 11 March 1999 (11.03.99)		
Applicant's or agent's file reference DFN-025PC		**IMPORTANT NOTICE**
International application No. PCT/US98/18432	**International filing date** (day/month/year) 04 September 1998 (04.09.98)	**Priority date** (day/month/year) 05 September 1997 (05.09.97)
Applicant DANA-FARBER CANCER INSTITUTE et al		

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   EP,JP,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   CA

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   11 March 1999 (11.03.99) under No. WO 99/11281

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT IB 301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18432

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 38/02, 38/10
US CL :514/2, 14
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 14

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG
search terms: mastoparan, G protein, septic shock, endotoxic shock, sepsis, antibiotic

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,492,898 A (BERTICS ET AL) 20 February 1996 (20/02/96), see column 2, lines 13-18, column 6, lines 28-34, column 9, line 67 - column 10, line 6.	1-3, 6-14
X	US 5,589,568 A (HIGASHIJIMA ET AL) 31 December 1996 (31/12/96), abstract, column 7, lines 1-20.	1-9, 11-16
X	CABEZA-ARVELAIZ et al. Cholera and Pertussis Exotoxins Protect Mice Against the Lethal Schwartzman Reaction and Antagonize the Effects of Lipopolysaccharide on Second Messenger Systems. Lymphokine Research. 1990, Volume 9, Number 2, pages 125-135, especially page 126, lines 12-17 and 24-31, page 128, Table 1, page 133, lines 6-17.	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 02 NOVEMBER 1998	Date of mailing of the international search report 19 NOV 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEFFREY E. RUSSEL for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18432

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCTOR et al. Protection of mice from endotoxic death by 2-methylthio-ATP. Proceedings of the National Academy of Science USA. June 1994, Volume 91, pages 6017-6020, especially page 6020, column 1, Table 1 and first full paragraph.	1-3, 6-9, 11-14
X	SOLOMON et al. G proteins regulate LPS mediated cytokine release. Clinical Infectious Diseases. August 1997, Volume 25, Number 2, page 370, Abstract Number 83.	1-9, 11-16

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference DFN-025PC	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US98/18432	International filing date *(day/month/year)* 04 SEPTEMBER 1998	Priority date *(day/month/year)* 05 SEPTEMBER 1997
International Patent Classification (IPC) or national classification and IPC IPC(6): A61K 38/02, 38/10 and US Cl.: 514/2, 14		
Applicant DANA-FARBER CANCER INSTITUTE		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☒ Certain observations on the international application

Date of submission of the demand 05 APRIL 1999	Date of completion of this report 21 OCTOBER 1999
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEFFREY E. RUSSEL Telephone No. (703) 308-0196 JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/18432

I. Basis of the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments)*:

[X] the international application as originally filed.

[X] the description, pages 1-15 , as originally filed.
pages NONE , filed with the demand.
pages NONE , filed with the letter of _____.
pages _____ , filed with the letter of _____.

[X] the claims, Nos. 1-17 , as originally filed.
Nos. NONE , as amended under Article 19.
Nos. NONE , filed with the demand.
Nos. NONE , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.

[X] the drawings, sheets/~~fig~~ 1-9 , as originally filed.
sheets/~~fig~~ NONE , filed with the demand.
sheets/~~fig~~ NONE , filed with the letter of _____.
sheets/~~fig~~ _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

[X] the description, pages NONE .
[X] the claims, Nos. NONE .
[X] the drawings, sheets/~~fig~~ NONE .

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/18432

V. Reasoned statement under Article 35(2) with regard to n velty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	10, 17	YES
	Claims	1-9, 11-16	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-17	NO
Industrial Applicability (IA)	Claims	1-17	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-3, 6-9, and 11-14 lack novelty under PCT Article 33(2) as being anticipated by Bertics et al. Bertics et al teach reducing the deleterious effects of endotoxin and endotoxic shock including LPS-induced shock by administering a 2-alkylthioadenosine-5'-nucleotide which blocks both LPS-induced GTPase activity and TNF production. See, e.g., column 2, lines 13-18; column 6, lines 28-34; and column 9, line 67 - column 10, line 6.

Claims 10 and 17 lack an inventive step under PCT Article 33(3) as being obvious over Bertics et al. Bertics et al is applied in the immediately preceding paragraph. Bertics et al teach that antibiotic treatment is a current therapy for treating gram negative bacteria, but does not teach the combination of an antibiotic with the nucleotide. It would have been obvious to one of ordinary skill in the art at the time Applicant's invention was made to use a combination of the antibiotic and nucleotide taught by Bertics et al to treat gram negative bacteria infection because it is prima facie obvious to use a combination of treatments where each treatment has been used individually to treat the same disease and where there is no indication of negative interaction between the treating agents.

Claims 1-9 and 11-16 lack novelty under PCT Article 33(2) as being anticipated by Higashijima et al. Higashijima et al teach methods and compositions for modulating the action of G proteins. Mastoparan analogs are used. See, e.g., the abstract and column 7, lines 1-20. With respect to claims 1-9, because the same active agent is being administered to the same subject by the same method steps, inherently septic shock will be prevented in Higashijima et al to the same extent claimed by Applicant. With respect to claims 11-16, a suggested use limitation does not impart novelty or non-obviousness to a composition claim where the composition is otherwise taught or suggested by the prior art.

Claims 1-9 and 11-16 lack novelty under PCT Article 33(2) as being anticipated by Cabeza-Arvelaiz et al. Cabeza-Arvelaiz et (Continued on Supplemental Sheet.)

Supplemental Box
(T be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
al teach the use of pertussis toxin and cholera toxin to inhibit the effects of LPS. The toxins antagonize LPS activation of G proteins. See, e.g., the Abstract; page 126, lines 12-17 and 24-31; page 128, Table 1; and page 133, lines 6-17. The toxins constitute analogs of mastoparan because of the toxins have the same function and effect as mastoparan in treating or prevent septic shock, and because the claims do not set forth any structural limitations on what constitutes an analog of mastoparan.

Claims 10 and 17 lack an inventive step under PCT Article 33(3) as being obvious over Cabeza-Arvelaiz et al. Cabeza-Arvelaiz et al is applied in the immediately preceding paragraph. Cabeza-Arvelaiz et al teaches that antibiotic treatment is a current therapy for LPS-induced shock, but does not teach the combination of an antibiotic with the toxin. It would have been obvious to one of ordinary skill in the art at the time Applicant's invention was made to use a combination of the antibiotic and toxin taught by Cabeza-Arvelaiz et al to treat gram negative bacteria infection because it is prima facie obvious to use a combination of treatments where each treatment has been used individually to treat the same disease and where there is no indication of negative interaction between the treating agents.

Claims 1-3, 6-9, and 11-14 lack novelty under PCT Article 33(2) as being anticipated by Proctor et al. Proctor et al teach administration of 2-methylthio-ATP to protect mice from endotoxic death. The 2-methyl-ATP antagonizes LPS activation of G proteins. See, e.g., the Abstract and page 6020, column 1, Table 1 and first full paragraph.

Claims 11 and 14-16 lack novelty under PCT Article 33(2) as being anticipated by Solomon et al. Solomon et al teach G protein agonists or antagonists such as mastoparan. See the entire abstract. Note that a suggested use limitation does not impart novelty or non-obviousness to a composition claim where the composition is otherwise anticipated by or obvious over the prior art.

Claims 1-17 meet the criteria set out in PCT Article 33(4). The claimed invention would have been expected to have industrial applicability in the treatment or prevention of septic shock.

---------- NEW CITATIONS ----------
NONE

VIII. Certain observati ns n the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 5 and 16 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because the claims are indefinite for the following reason(s): The term "analog" in claims 5 and 16 is indefinite because it is not defined either in the description or the art. It is not clear what degree of functional or structural similarity is necessary for one compound to be considered an analog of another compound.

THE USE OF AGENTS WHICH BIND G PROTEINS FOR TREATING SEPTIC SHOCK

Background of the Invention

Septic shock (also known as sepsis) causes more than 150,000 deaths annually in the United States. Sepsis is defined as a clinical disorder whose symptoms may include well defined abnormalities in body temperature, heart rate, breathing rate, white blood cell count, hypotension, organ perfusion abnormalities, and multiple organ dysfunction. There are several causes of sepsis including bacterial (either gram negative or gram positive), fungal and viral infections, as well as non-infective stimuli such as multiple trauma, severe burns, organ transplantation and pancreatitis.

Septic patients usually die as a result of poor tissue perfusion and injury followed by multiple organ failure. It is well recognized that many of the responses that occur during septic shock are initiated by bacterial endotoxin, a glycolipid antigen present on the surface of gram negative bacteria. This endotoxin (also referred to herein as lipopolysacchride or LPS) is released upon the deatn or multiplication of the bacteria and is known to activate monocytes/macrophages or endothelial cells causing them to produce various mediatior molecules such as toxic oxygen radicals, hydrogen peroxide, tumor neurosis factor-alpha (TNFα), and interleukin (IL-1, IL-6, and IL-8). Theses cellular and humoral inflammatory mediators evoke septic shock with symptoms ranging from chills and fever to circulatory failure, multiorgan failure, and death.

The impact of sepsis is particularly devastating to patients with compromised cardiac and hepatic function and to immunocompromised patients. Patients at high risk are elderly, chemothearpy patients and those requiring surgery or invasive instrumentation. The current therapy of antibiotics and hemodynamic support has not proven to be successful. An improved method for treating or preventing septic shock would be of great value.

The major LPS receptor for monocytes/macrophages is the glycosylphosphatidylinositol (GPI) anchored glycoprotein CD14. It is the interaction of LPS with the LPS receptor CD14 that initiates the cascade of signaling events that cause cytokine gene transcription. The precise mechanism through which LPS interacts with CD14 is unknown. Much of the controversy regarding the role of CD14 in LPS-induced signal transduction and cytokine production stems from the fact that CD14 is attached to the cell membrane by a glycosylphosphatidylinositol (GPI)-anchor and contains neither transmembrane nor cytoplasmic amino acid sequences. As such, CD14 cannot interact with signal transduction molecules in the same way as transmembrane receptors. Recently, it has been demonstrated that GPI-anchored proteins expressed on many cell

types can physically interact with lipid-linked signal transduction molecules, but the functional consequences of these interactions remain unresolved (Stefanova et al., *Science*, 254: 1016-1018, 1991; Shenoy-Scaria et al., *Mol Cell Biol.*, 13: 6385-6392, 1993; Solomon et al., *Proc. Nat. Acad. Sci.*, 93: 6053-6058, 1996).

Although the precise mechanism through which LPS binding to CD14 leads to cell activation is not known, it has been demonstrated that this interaction is enhanced by the serum factor LPS-binding protein (LBP) (Shumann et al., *Science*, 249: 1429-1432, 1990; Hailman et al., *J. Exp. Med.*, 179: 269-277, 1994). The interaction of LPS/LBP with CD14 causes the exchange of LPS with lipids in target membranes (Wurfel et al., *J. Exp. Med.*, 181: 1743-1754, 1997; Yu et al., *J. Clin. Med.*, 99: 315-324, 1997; Wurfel et al., *J. Immunol.*, 158: 3925-3934, 1997). It has been suggested that this lipid transfer is responsible for LPS-induced signal transduction. The rate of the exchange reaction depends on the lipid composition of the target membranes, which has led to speculation that CD14 functions only to direct LPS insertion into particular membrane domains (Hailman et al., *J. Exp. Med.*, 179: 269-277, 1994; Wurfel et al., *J. Immunol.*, 158: 3925-3934, 1997). While the mechanism that leads to LPS-induced signal transduction has not been demonstrated, it is known that monocyte activation by LPS leads to the phosphorylation of p38 mitogen activated protein kinase (MAPK), and production of inflammatory cytokines (i.e., TNF-α, IL-6) (Sweet, M.J. and Hume D.A., *J. Leuk. Biol.*, 60: 8-26, 1996).

Summary of the Invention

The present invention is based, at least in part, on the discovery that CD14 on monocytes/macrophages physically interacts with heterotrimeric G proteins and, in particular, that such G proteins specifically regulate LPS-induced mitogen activated protein (MAP) kinase activation and cytokine production in human monocytes/macrophages. This invention is further based on the discovery that agents which bind G proteins, such as G protein binding peptides, inhibit G protein signal transduction to thereby treat or prevent septic shock *in vivo*.

Accordingly, this invention provides compositions and methods for treating or preventing septic shock in a subject at risk of developing septic shock The method comprises administering an effective amount of an agent which binds G protein such that septic shock is treated or prevented in the subject. The agents which bind G protein are useful for both proplylactic and/or therapeutic treatments of septic shock.

The invention also pertains to compositions for treating or preventing septic shock in a subject which include an effective amount of an agent which binds G protein

and, optionally, an antibiotic. The composition can further include a pharmaceutically acceptable carrier.

The present invention also provides methods for using agents which bind G proteins in combination with other agents and/or treatment regimens (e.g., antibiotics, intravenous fluids, cardiovascular and respiratory support) to prophylactically and/or therapeutically treat a subject for septic shock. Other aspects of the invention include packaged agents which bind G proteins and instructions for using such agents for treatment of septic shock.

Brief Description of the Drawings

Figures 1A and 1B are gels depicting the association of CD14 with src kinases and heterotrimeric G proteins. Figure 1A shows that CD14 is associated with tyrosine phosphorylated proteins. Figure 1B shows that CD14 is associated with various src kinases and heterotrimeric G protein α subunits.

Figure 2 depicts the inhibition of IL-6 production from LPS-stimulated PMBCs by mastoparan. Figure 2 shows that mastoparan but not its inactive analogue (MAS-17) inhibits LPS-induced cytokine production.

Figures 3A and 3B depict the inhibition of cytokine production from LPS-stimulated monocytes by mastoparan. Figure 3A shows a dose-dependent inhibition of IL-6 and TNF-α production in human monocytes by mastoparan. Figure 3B shows inhibition of LPS-, but not PMA-, induced cytokine production from human monocytes by mastoparan. The experiments shown are representative of 6 different assays with similar results.

Figure 4 shows that mastoparan only inhibits CD14-dependent LPS-induced signal transduction in U373-CD14 tranfected cells.

Figures 5A and 5B depict the inhibition of phosphorylation of p38 MAP kinase in human monocytes and PMBC by mastoparan. Figure 5B shows that mastoparan reduces LPS-induced p38 MAP kinase activity.

Figure 6 is a gel depicting the effect of LPS and mastoparan on MAP kinase activation in human monocytes. Figure 6 shows specific inhibition of LPS-induced Erk kinase activation by mastoparan.

Detailed Description of the Invention

The present invention pertains to a method for treating or preventing septic shock in a subject by administering to the subject an effective amount of an agent which binds to G protein, such that septic shock in the subject is treated or prevented. Septic shock is commonly associated with bacterial infection in a surgical setting or with

immunocompromised subjects, and is typically characterized by abnormalities in body temperature, heart rate, blood pressure and breathing which can progress to spontaneous clotting in blood vessels, multiple organ failure and death. The present invention also pertains to a composition for treating or preventing septic shock in a subject which includes an effective amount of an agent which binds G protein to treat or prevent septic shock in the subject. The composition can further include an antibiotic and/or a pharmaceutically acceptable carrier.

The term "septic shock" or "sepsis" refers to a clinical disorder whose symptoms may include well defined abnormalities in body temperature, heart rate, breathing rate, white blood cell count, hypertension, organ perfusion abnormalities, and multiple organ dysfunction. It may be caused by bacterial (either gram negative or gram positive), fungal, viral or other infection, as well as by non-infective stimuli such as multiple trauma, severe burns, organ transplantation and pancreatitis. Septic shock is commonly caused by "gram-negative" endotoxin-producing aerobic rods --*Escherichia coli, Klebsiella pneumoniae, Proteus* species, *Pseudomonas aeruginosa* and *Salmonella*. Septic shock involved with gram negative bacteria is referred to as "endotoxic shock". A significant portion of the peripheral responses occurring during septic shock are initiated by endotoxin (also referred to herein as lipopolysaccharide or "LPS"), an outer-membrane component of gram-negative bacteria which is released upon the death or multiplication of the bacteria. The manner in which LPS evokes its effects is by binding to cells such as monocytes/macrophages or endothelial cells and triggering them to produce various mediators, such as oxygen radicals, hydrogen peroxide, tumor necrosis factor-alpha (TNF-α), and various interleukins (IL-1, IL-6, and IL-8). Gram-positive bacteria, particularly pneumococcal or streptococcal, may produce a similar clinical syndrome as endotoxic shock. Thus, as used herein, the term "endotoxic shock" refers to septic shock involved with gram negative and/or gram positive bacteria.

Whatever the offending agent, the septic shock affects the peripheral circulation of a subject. The affects of septic shock include direct toxic injury to the subject which may induce arteriolar vasodilation and pooling of blood and elevated capillary pressure leading to the escape of plasma water into the interstitial compartment, further impinging on the circulating blood volume of the subject. In addition, septic shock leads to an inflammatory-immune reaction which results in the release of vasodilators in the subject, such as histamine and complement fractions, further reducing the effective circulating volume. Platelet activation and the formation of thromboxane A_2 may add an element of platelet aggregation, with activation of the clotting sequence and the induction of disseminated intravascular coagulation in the subject. The overall effect of septic shock in the subject is a decrease in blood volume.

The language "treat for septic shock" is intended to cover therapeutic and/or prophylactic treatments. The agents which bind G proteins can be used to protect a subject from damage on injury caused by septic shock or can therapeutically treat a subject prior to the onset of septic shock. For therapeutic treatment, agents which bind G protein can treat or reduce the damage or injury caused by septic shock in a subject having one or more symptoms of septic shock. Symptoms of septic shock in a subject include tremors, fever, falling blood pressure, rapid breathing, rapid heart beat, and skin lesions. Within hours or days, these symptoms can progress to spontaneous clotting in blood vessels, severe hypotension and multiple organ failure. The term "treat" as used herein refers reducing or preventing at least one symptom associated with sepsis. The treatment of a subject need not be complete restoration to the subjects previous state, but rather can be an amount sufficient to prevent mortality of the subject.

For prophylactic treatments, agents which bind G proteins are administered prior to the onset of symptoms of septic shock to prevent or inhibit septic shock in subjects at risk of developing or susceptible to septic shock. Subjects at risk of developing septic shock include the elderly, immunocompromised patients (e.g., chemotherapeutic and HIV patients), patients requiring surgery or invasive instrumentation, and patients with compromised cardiac and hepatic function. Administering to a subject an agent which binds G protein in these types of settings can be used to decrease the susceptibility of these patients to septic shock.

The term "subject" is intended to include mammals having septic shock, including one or more of the symptoms related to sepsis. Examples of such subjects include humans, dogs, cats, pigs, cows, horses, rats, and mice.

The term "administering" is intended to include routes of administration which allow the agent to perform its intended function of treating or preventing septic shock by binding to G protein. Examples of routes of administration which can be used include parental injection (e.g., subcutaneous, intravenous, and intramuscular), intraperitoneal injection, oral, inhalation, and transdermal. The injection can be bolus injections or can be continuous infusion. Depending on the route of administration, the agent can be coated with or disposed in a selected material to protect it from natural conditions which may detrimentally effect its ability to perform its intended function. When the agent is a peptide, such as mastoparan or analog thereof, the peptide can be modified at one or more of its termini to protect the peptide from degradation. Methods of protecting peptides from degradation are disclosed in U.S Patent No. 5,589,568 which is incorporated herein by reference. The agent can be administered with other agents and/or with a pharmaceuticlly acceptable carrier. Further, the agent can be administered as a mixture of agents which bind G proteins, which also can be coadministered with a

pharmaceutically acceptable carrier. The agent can be administered prior to the onset of septic shock or after the onset of septic shock.

The language "pharmaceutically acceptable carrier" is intended to include substances capable of being coadministered with the G protein binding agent, and which allows the agent to perform its intended function of treating septic shock or preventing septic shock. An example of such a carrier is saline. Any other conventional carrier suitable for use with the G protein binding agent also fall within the scope of the present invention.

The language "effective amount" of an agent which binds G protein is that amount necessary or sufficient to treat or prevent septic shock, e.g. prevent at least one of the various symptoms of septic shock, or that amount necessary to reduce the pathogenesis of septic shock. The effective amount can vary depending on such factors as the size and weight of the subject, the type of illness, the severity of the symptoms, or the particular agent used. One of ordinary skill in the art would be able to study the aforementioned factors and make the determination regarding the effective amount of G protein binding agent without undue experimentation.

The regimen of administration can affect what constitutes an effective amount. G protein binding agents can be administered to the subject either prior to or after the onset of septic shock. Further, several divided dosages, as well as staggered dosages, can be administered daily or sequentially, or the dose can be continuously infused or can be a bolus injection. Further, the dosages of the G protein binding agent(s) can be proportionally increased or decrease as indicated by the exigencies of the therapeutic or prophylactic situation.

G proteins (guanine nucleotide binding regulatory proteins) are important to regulatory mechanisms operating in all human cells. Impairment of their function can perturb the cell's response to hormonal signals and adversely affect many intracellular metabolic pathways, thus contributing to the development and maintenance of a wide variety of disease states. When functioning normally, G proteins act as an integral part of the signal transducing mechanism by which extracellular hormones and neurotransmitters convey their signals through the plasma membrane of the cell and thus elicit appropriate intracellular responses. In its simplest terms, the signal transducing mechanism of G protein can be said to comprise three distinct components. A receptor protein with an extracellular binding site specific for a given agonist; a membrane-bound effector protein that when activated catalyzes the formation of facilitates the transport of an intracellular second messenger, an example is adenylate cyclase which produces cyclic AMP (cAMP); and a third protein which functions as a communicator between

these two. G proteins fulfill this vital role as communicator in the generation of intracellular responses to extracellular hormones and agonists.

G proteins are composed of three polypeptide subunits, namely G alpha (α), G beta (β), and G gamma (γ). The conformation of each subunit and their degree of association changes during the signal transducing mechanism. These changes are associated with the hydrolysis of the nucleotide GTP to form GDP and P sub i (GTPase activity). The binding sites for GTP, GDP and the GTPase catalytic site reside in the α subunit.

The G protein cycle which occurs each time a signal is conveyed across the membrane can be summarized as follows: 1) in an unstimulated cell the G proteins are found in the resting state in which α, β and γ are complexed together and GDP is bound to G α, 2) the binding of an appropriate hormone or agonist to the receptor changes its conformation and causes it to activate the G protein by displacing GDP and allowing GTP to bind (this is the rate-limiting step of the G protein cycle), 4) when GTP is bound to G α it may dissociate from the β and γ complex and is able to bind to, and activate, adenylate cyclase which releases cAMP into the cytoplasm, 5) GTP is then hydrolysed to GDP and the cycle is complete.

A further attribute inherent in this system is that it allows several different receptors to interact with a signal-generating enzyme. Some act in such a way to activate the enzyme and some to inhibit it. This involves distinct alpha subunits G sub s alpha (stimulatory) and G sub i alpha (inhibitory) that combine with the same beta gamma complex to form stimulatory or inhibitory G proteins. An example of a receptor that interacts with G sub i to lower the concentration of cAMP is the alpha sub 2-adrenergic receptor. The integration of the signals from G sub s and G sub i is one of the ways in which the level of cAMP in the cell can be modulated in response to several different extracellular agonists. The present invention is based on, at least in part, the demonstration of both a physical and functional coupling of CD14 to G proteins and the involvement of G proteins in the regulation of LPS-induced signal transduction.

The term "agent which binds to G protein" or "G protein binding agent" refers to an agent which binds to G protein and inhibits G protein signal transduction, such as a small molecule, compound, drug, polypeptide, or peptide. Preferred G protein binding agents bind a G α subunit. Particularly preferred agents are cell permeable agents. A particularly preferred G protein binding agent for treating bacterial sepsis is a peptide. Examples of such peptides include mastoparan and analogs thereof. Mastoparan is a permeable, amphiphilic peptide that binds Gi and Go heterotrimeric G proteins (Higashijima et al., *J. Biol. Chem.*, 263: 6491-6494, 1988; Higashijima et al., *J. Biol. Chem.*, 265: 14176-14186, 1990; Higashijima et al., *J. Biol. Chem.*, 265: 14176-14186,

1991). Mastoparan is the prototype of a family of peptide toxins, collectively known as mastoparans, that form amphiphilic alpha helices. Mastoparan has been shown to stimulate guanine nucleotide exchange by G proteins in a manner similar to that of G protein-coupled receptors. The mastoparans of the present invention can be naturally occurring mastoparans, or analogs thereof, which are known in the art and are described in U.S. Patent 5,589,568, incorporated herein by reference. Mastoparan can be synthesized and purified as described by Saito (*Chem. Pharm. Bull.*, 32: 2187-2193, 1984) incorporated herein by reference, or alternately can be purchased from Sigma (St. Louis, MO). The peptides of the present invention can be prepared by standard peptide synthesis technology (e.g., Merrifield, *J. Am. Chem. Soc.*, 88: 2149-2154, 1963; Houghton et al., *Int. J. Pept. Protein Res.*, 16: 311-320, 1980; Eler, *J. Biochem.*, 145: 157-162, 1984; *PNAS USA*, 82: 5131-5135, 1988), for example, by using a solid phase peptide synthesizer and purified by reverse phase HPLC. Such methods of peptide synthesis and purification are known in the art.

Agents which bind G proteins and inhibit G protein signal transduction can be identified by use of one or more assays known to those skilled in the art (for a review see Kaziro, Y. et al. (1991) *Ann. Rev. Biochem.*, 60: 349-400; and Neer, E. J., (1995) *Cell* 80: 249-257) or described herein. For example, G protein binding agents which inhibit G protein signal transduction can be identified by their ability to inhibit the production of cytokines (such as IL-6 and TNF-α) when cells (such as monocytes or macrophages) are contacted with the agent and a G protein stimulator, such as LPS. Alternatively, an agent which binds G protein can be identified by its ability to inhibit MAP kinase activation, under conditions as described herein. The *in vivo* efficacy of an agent which binds G protein and inhibits G protein signal transduction can be determined by the ability of such an agent to protect rats from LPS-induced lethal endotoxic shock as described herein.

The present invention also pertains to compositions and methods for treating a subject having septic shock or susceptible to septic shock. The composition contains an effective amount of an agent which binds G protein and a pharmaceutically acceptable treatment solution.

The present invention further pertains to the use of agents which bind G proteins together with an antibiotic for prophylactic and/or therapeutic treatments of septic shock. The invention also pertains to compositions for treating a subject for septic shock which include an effective amount of the antibiotic and an agent which binds G protein in a pharmaceutically acceptable carrier.

The present invention further pertains to the use of G protein binding agents in conjunction with other agents or regimens for therapy or prophylactic treatment of septic

shock. Some specific examples of other agents or regimens which can be administered to the subject to treat septic shock include antibiotics, intravenous fluids and cardiovascular and respiratory support.

The invention is further illustrated by the following examples which in no way should be construed as being further limiting. The contents of all references, pending patent applications, published patent applications, and issued patents cited throughout this application are hereby incorporated by reference. It should be understood that the animal models used throughout the examples are accepted animal models and that the demonstration of efficacy in these animal models is predictive of efficacy in humans.

EXAMPLES

The following materials and methods were used throughout the examples.

Isolation of human PBMC and monocytes.

Freshly isolated human peripheral blood mononuclear cells (PBMC) and monocytes were obtained from leukopaks (discarded leukocyte from platelet donations). The cells were fractionated on FICOLL-HYPAQUE™ gradients, washed, treated with tris-buffered NH_4Cl to eliminate RBCs and washed to obtain PMBCs. Monocytes were obtained by depleting the PBMCs of T cells and NK cells by negative selection asking standard techniques. T cells and NK cells were removed by treatment with anti-CD3 and anti-CD2 monoclonal antibodies followed by goat anti-mouse Ig conjugated magnetic beads at a 10:1 bead:cell ratio. The monocyte preparations were at least 80-85% monocytes, as determined by anti-CD14 staining and forward and slide light scatter analysis using a FACScan (Becton-Dickenson, Elmhurst, IL). Less than 2% of the contaminating cells in the monocyte preparation were T cells and no NK cells could be detected. Monocytes were maintained in Ham's F-12 10% FCS, L-Glutamine and penacillin/streptomyocin at 37°C in 5% CO_2.

Cell Lines

U373 cell CD14 transfectants (U373-CD14) were maintained in EMEM supplemented with 10% FCS, L-Glutamine and penicillin/streptomyocin at 37°C in 5% CO_2.

Immunoprecipitation, *in vitro* kinase assay, and reimmunoprecipitation.

Assays were performed as described in Solomon et al., *Proc. Nat. Acad. Sci, 93*:6053-6058 (1996). Briefly, cells were washed 3 times in cold buffered saline and were lysed on ice for 30 minutes in lysis buffer [0.5% NP40; 300 mM NaCl; 50 mM Tris pH 7.6; 0.15 u/ml aprotinin; 10 mM Iodoacetimide; 5 mM EDTA; 1 mM Na_3VO_4 10 μg/ml leupeptin; 1 mM PMSF]. Insoluble debris was removed by microcentrifugation and the lysates were precleared with 100 μl (10% w/v) rabbit anti-mouse coated protein A sephrose beads (1 mg/ml) followed by 200 μl (10% w/v) protein A sephrose beads. The lysates were then incubated for two hours at 4°C with monoclonal antibodies previously bound to protein A sephrose beads. After 2 hours, the beads were washed 4 times in lysis buffer and once in Kinase buffer [25 mM hepes, 1 mM $MnCl_2$ and 100 μM Na_3VO_4] and the immunoprecipitates were then resuspended in 50 μl kinase buffer with 20μCi[γ^{32}P]ATP (New England Nuclear, Boston, MA) and incubated for 15 minutes at room temperature. The samples were washed 4 times in lysis buffer with 15 mM EDTA. Samples were then eluted in 0.5% SDS at 70°C for 3 minutes or boiled in 1% SDS for 5 minutes and diluted 10 fold with cold lysis buffer. Samples were analyzed by SDS-PAGE or reimmunoprecipitated with various monoclonal or polyclonal antisera (e.g., rabbit) prior to SDS-PAGE analysis. Reimmunoprecipitated samples were boiled in reducing Laemmli sample buffer, and subjected to electrophoresis through a 10% SDS-PAGE gel.

Cytokine production

Freshly isolated human monocytes, human PBMC and U373-CD14 cells were incubated with 10 ng/ml LPS (*E coli* 0111:B4, Sigma, St. Louis, MO), 100 ng/ml PMA or were untreated in supplemented RPMI [10% FCS; 2 mM L-glutamine and penicillin/streptomycin] with or without various concentrations of mastoparan for 18 hours at 37°C in 24 well tissue culture dishes. IL-6 and TNF-α levels were determined by ELISA (Endogen Inc., Boston, MA) of supernatants harvested at 4 hours (for TNF-α) and at 18 hours (for IL-6) after LPS stimulation.

Immunoblotting

For detection of p38, phosphorylated p38 and phosphorylated Erk kinases in monocyte lysates, monocytes were subjected to various treatments and lysed in boiling reducing Laemmli sample buffer. The lysates were subjected to electrophoresis through a 10% SDS PAGE gel and were then transferred to nitrocellulose (NC). After washing twice with TBS-Twwen 20(0.1%) the NC was placed in a solution of Ponceau S dye to ensure equal loading and left in blocking buffer [1x TBS; 0.1% TBS; 5% milk] for 1

hour. After blocking and washing, the NC was incubated with anti-p38 N + C terminus (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 2-3 hours or with anti-phosphorylated p38 and Erk antibodies (New England Biolabs, Inc., Beverly, MA) for 18 hours. Membranes were washed three times in TBS-Tween 20 and were incubated for 30 minutes with horseradish peroxidase conjugated donkey-α-rabbit antibody in blocking buffer (Amersham Corp., Arlington Heights, IL). Membranes were washed an additional six times [3 x TBS-Tween-20; 3 x TBS], and were developed by exposure to ECL chemicals (Amersham corp., Arlington Heights, IL) and visualized by exposure to film.

For detection of p38 in nuclear extracts, cells after a variety of treatments were subject to lysis and nuclear extraction as described in Lerder et al., *J. Immunol. 152*:77-86 (1994). The nuclear extracts were subjected to protein quantitation by Micro BCA assay (Pierce, Rockford, IL) using a BSA standard. The details of the blotting procedure were the same as above except that 5-10 ng of extract was used.

Lethal endotoxin shock

Wistar rats (200 g) were obtained from Charles River Laboratories. Rats were treated with 3mg/kg mastoparan by intravenous injection in the tail vein, immediately followed by 15 mg/kg lead acetate and 5 μg/kg LPS 0111:B4 intravenously. Mortality was assessed up to 96 hours following LPS treatment. Mortality frequency was compared by Fisher exact test and statistical analysis was performed using Yates corrected Chi square test..

Example 1. Association of CD14 with G Proteins Following LPS Stimulation

To elucidate the mechanism of LPS-induced signal transduction mediated through CD14, CD14 was immunoprecipitated from freshly isolated human monocytes and *in vitro* kinase assays performed to asses the association of CD14 with phosphorylated proteins.

Results from these assays revealed the presence of multiple tyrosine phosphorylated species which coimmunoprecipitated with CD14 (See Figure 1A). Reimmunoprecipitation of the product of these assays with an anti-phosphotyrosine specific antibody indicated that all the major phosphorylated species were tyrosine phosphorylated. Immunoprecipitation of the products of the *in vitro* kinase assay with heterosera and various monoclonal antibodies recognizing src family tyrosine kinases indicated that in human monocytes fyn, lyn and fgr src family kinases were all present in substantial quantities and a small amount of hck was observed on overexposed

autoradiographs (Figure 1B). There was no evidence for lck in the immunoprecipitates from human monocytes.

In vitro kinase assays of immunoprecipitated CD14 also revealed the presence of a 40 kD tyrosine phosphorylated species (Figures 1A and 1B). This protein could be immunoprecipitated from the products of CD14 *in vitro* kinase assays with a pan-anti-G protein antiserum which recognizes the GTP binding site of a variety of G proteins, as described in Solomon et al., *Proc. Nat. Acad. Sci,* 93: 6065-6058 (1996), indicating that this species was a G protein. Immunoprecipitation of the products of the CD14 *in vitro* kinase assays with antisera specific for α subunits of the heterotrimeric G proteins indicated that this 40 kD protein consisted of a combination of heterometrimeric G protein α subunits of a small amount of Gi α1 and larger amounts of Gi α2, Gi α3 and Go α. In contrast, Gs α was not found in association with CD14 from the CD14 *in vitro* kinase assay. Similar patterns of heterometrimeric G proteins and src family kinases were found to coimmunoprecipitate with CD14 from CHO- and U373-CD14 transfectants and are similar to the patterns of phosphoproteins associated with other GPI-anchored proteins.

In addition, there was no evidence for the β or γ subunits of heterotrimeric G proteins in these immunoprecipitates. This may be due to the documented dissociation of these subunits from the α subunit during detergent lysis (Chang et al., *J. Cell Biol.*, 126: 127-138, 1994) or simply be an indication that these subunits are not phosphorylated in the *in vitro* kinase reactions.

Thus, the fact that CD14 immunoprecipitates contained Gi and Go heterodimer G proteins suggests that G proteins may be involved in LPS-induced signaling.

Example 2. *In Vitro* Effect of Mastoparan on G Protein Signal Transduction

To investigate the functional consequences of the G protein/CD14 association, human cells (i.e., monocytes and PBMCs) and U373 cell transfectants expressing CD14 were treated with mastoparan (a cell permeable, amphiphilic peptide that binds Gi and Go heterotrimeric G proteins) and LPS to determine the effect on LPS-induced cytokine production.

Initially, the effect of mastoparan (QCB, Hopkinton, MA) and its inactive analogue, MAS-17 (QBC) on cytokine production from human PBMCs were tested. Freshly isolated PBMCs were treated with LPS and/or peptides (mastoparan or the MAS-17 control peptide) and IL-6 levels were measured in the tissue culture supernatants of the cells (Figure 2). PBMCs produced IL-6 in response to LPS, while neither mastoparan nor MAS-17 stimulated IL-6 production from the PBMCs. Mastoparan was a potent inhibitor of LPS-induced IL-6 production from the LPS

stimulated PBMCs, while MAS-17 had no effect on cytokine production. The effect of mastoparan on cytokine production was dose-dependent, and at a concentration of 13.3 μM mastoparan totally ablated LPS-induced IL-6 production from the PBMCs.

In order to determine if the effect of mastoparan on LPS-induced cytokine production was due to a direct effect on monocytes, a highly enriched monocyte population was tested for the effect of LPS and mastoparan on cytokine production from these cells. Freshly isolated human monocytes were treated with mastoparan and LPS, after which IL-6 and TFN cytokine levels were measured in the tissue culture supernatants of the cells (Figure 3A). Untreated monocytes did not produce detectable levels of cytokines, verifying that the isolation procedure had not activated these cells. LPS caused a dose-dependent stimulation of cytokines from isolated monocytes, whereas mastoparan induced neither IL-6 nor TNF production from these cells. When mastoparan was used in conjunction with LPS, cytokine production was diminished. Concentrations of mastoparan as low as 1.67 μM caused dramatic reductions in both IL-6 and TNF production in monocytes stimulated with LPS. Mastoparan at 13.34 μM concentrations totally ablated LPS-induced cytokine production from these cells. Mastoparan had little effect on cytokine production from PMA stimulated cells (Figure 3B), indicating the specificity of mastoparan action, and lack of mastoparan toxicity. Mastoparan had no effect on cell viability as measured by trypan blue uptake even after 36 hours of continuous mastoparan incubation.

In addition, the effect of mastoparan on LSP-induced cytokine production from an LPS responsive, CD14 transfected cell line was determined. LPS treatment of U373-CD14 transfectants induced an LPS dose-dependent production of IL-6 (Figure 4). At low concentrations of LPS (10 ng/ml-100 ng/ml) LPS-induced IL-6 responses were completely inhibited by treatment of the U373-CD14 cells with an anti-CD14 monoclonal antibody. IL-6 production induced by 10 ng/ml LPS was also ablated by treatment of the cells with mastoparan, while at 100 ng/ml of LPS, mastoparan reduced IL-6 levels by approximately one third. At high concentrations of LPS (1 μg/ml) the IL-6 responses of these cells were not inhibited by treatment with the anti-CD14 monoclonal antibody. Thus, at high concentrations of LPS, U373 cells exhibit CD14-independent LPS induced cytokine responses. At LPS concentrations of 1 μg/ml, mastoparan was ineffective at reducing cytokine responses from these cells. Thus, CD14-independent LPS signals were not inhibited by mastoparan.

Thus, pharmacologic targeting of the same heterotrimeric G proteins which are associated with CD14 had a substantial and specific impact on LPS induction of cytokine production.

LPS stimulation of cells through CD14 leads to the induction of a MAP kinase signaling pathway involving the p38 MAP kinase, which has been shown to be specifically induced by LPS (Figures 5A and 5B). Because mastoparan had profound effects on cytokine production and p38 MAP kinase is involved in LPS induced-signaling, the effect of mastoparan on MAP kinase activation was evaluated. For full activation, p38 MAP kinase requires phosphorylation on both threonine and tyrosine residues (Raingeaud et al., *J. Biol. Chem.*, 270: 7420-7426, 1995). Detection of dual-phosphorylated p38 MAP kinase by monoclonal antibodies specific for the dual-phosphorylated form of p38 was used as a measure of p38 activation. Consistent with the effect of mastoparan on LPS-induced cytokine production, mastoparan reduced the LPS-induced phosphorylation of p38 MAP kinase in both monocytes and PBMC (Figure 5A). It also inhibited LPS-induced nuclear translocation of p38.

Since it had been previously demonstrated that LPS induces phosphorylation of Erk 1 and 2 MAP kinases in transformed macrophage cell lines (Weinstein et al., *J. Immunol.*, 151: 3829-3838, 1993; Weinstein et al., *J. Biol. Chem.*, 267: 14955-14962, 1992), LPS-induced activation of ERK 1 and 2 and the effect of mastoparan on the activation of these kinases in normal human monocytes was determined (Figure 6). Only minimal phosphorylation of Erk 1 or 2 as seen in LPS-stimulated monocytes. The lack of substantial LPS-induced Erk kinase phosphorylation in freshly isolated monocytes is consistent with results obtained with other non-transformed human cells (Nick et al., *J. Immunol.*, 156: 4867-4875, 1996). In contrast to LPS, PMA induced substantial phosphorylation of Erk kinases. Interestingly, while mastoparan completely inhibited the Erk kinase activation induced by LPS, it had minimal effects on Erk kinase activation induced by PMA. This is consistent with the cytokine data, in so far as the effect of mastoparan was specific for the LPS signal transduction pathway and did not globally alter the ability to activate MAP kinases.

Example 3. *In Vivo* Effect of Mastoparan on G Protein Signal Transduction

The ability of mastoparan to inhibit cytokine production from human monocytes suggested that mastoparan may have efficacy in reducing LPS-induced pathology *in vivo*. To determine the effect of mastoparan *in vivo*, the effect of mastoparan on LPS-induced lethal endotoxic shock in rats was assessed.

Mastoparan did not have any toxic effects when used alone in the model system (data not shown). Mastoparan did significantly protect rats from LPS induced mortality (Table 1). These results demonstrate the importance of G protein-mediated events in endotoxic shock and that targeting heterotrimeric G proteins with pharmological agents which bind G proteins has therapeutic potential.

Table I

Group	Mortality		Total (%)
	14 hours	*24 hours*	
LPS	14/17	14/17	82.4%
LPS* + *Mastoparan	7/18	8/18	44.4%

Data were subjected to Yates corrected Chi square statistical analysis. The ability of mastoparan to protect rats from LPS-induced mortality was statistically significant $p<0.05$ at both 14 and 24 hours. The experiment was continued for 96 hours and the mortality remain unchanged from the 24 hour point.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments and methods described herein. Such equivalents are intended to be encompassed by the scope of the following claims.

What is claimed is

1. A method for treating or preventing septic shock in a subject comprising, administering to the subject an effective amount of an agent which binds G protein, such that septic shock in the subject is treated or prevented.

2. The method of claim 1, wherein the agent binds Gα subunit.

3. The method of claim 1, wherein the agent is a cell permeable agent.

4. The method of claim 3, wherein the agent is a peptide.

5. The method of claim 4, wherein the peptide is mastoparan or an analog thereof.

6. The method of claim 1, wherein the septic shock is endotoxic shock.

7. The method of claim 6, wherein the endotoxic shock is induced by gram negative bacteria.

8. The method of claim 1, wherein the endotoxic shock is induced by gram positive bacteria.

9. The method of claim 1, wherein the septic shock is LPS-induced shock.

10. The method of claim 1, further comprising administering an antibiotic to the subject.

11. A composition for treating or preventing septic shock in a subject comprising an effective amount of an agent which binds G protein to treat or prevent septic shock.

12. The composition of claim 11, wherein the agent binds Gα subunit.

13. The composition of claim 11, further comprising a pharmaceutically acceptable carrier.

14. The composition of claim 11, wherein the agent is a cell permeable agent.

15. The composition of claim 14, wherein the cell permeable agent is a peptide.

16. The composition of claim 15, wherein the peptide is mastoparan or an analog thereof.

17. The composition of claim 11, further comprising an antibiotic.

THE USE OF AGENTS WHICH BIND G PROTEINS FOR TREATING SEPTIC SHOCK

Abstract

The present invention provides for the use of G protein binding agents for prophylactic and/or therapeutic treatments of septic shock. The present invention provides methods of using agents which bind G protein to treat a subject having or susceptible to septic shock. The present invention further pertains to compositions for treating a subject for septic shock. The composition includes an effective amount of a G protein binding agent and, optionally, an antibiotic and a pharmaceutically acceptable carrier. Other aspects of the invention include packaged agents which bind G proteins for treating septic shock.


1°IP
CD14
Neg
IVK
2°IP
Anti-pY
Neg
Anti-pY
Neg
CD14
Neg
kD
112
84
53
35
29

FIGURE 1A

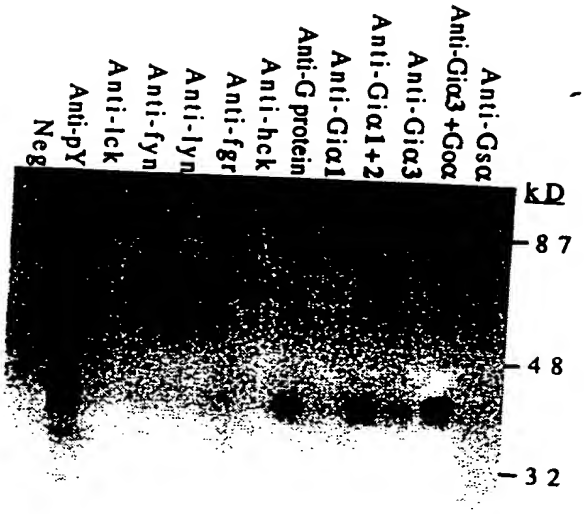

Neg
Anti-pY
Anti-lck
Anti-fyn
Anti-lyn
Anti-fgr
Anti-hck
Anti-G protein
Anti-Giα1
Anti-Giα1+2
Anti-Giα3
Anti-Giα3 +Goα
Anti-Gsα
kD
87
48
32

FIGURE1B

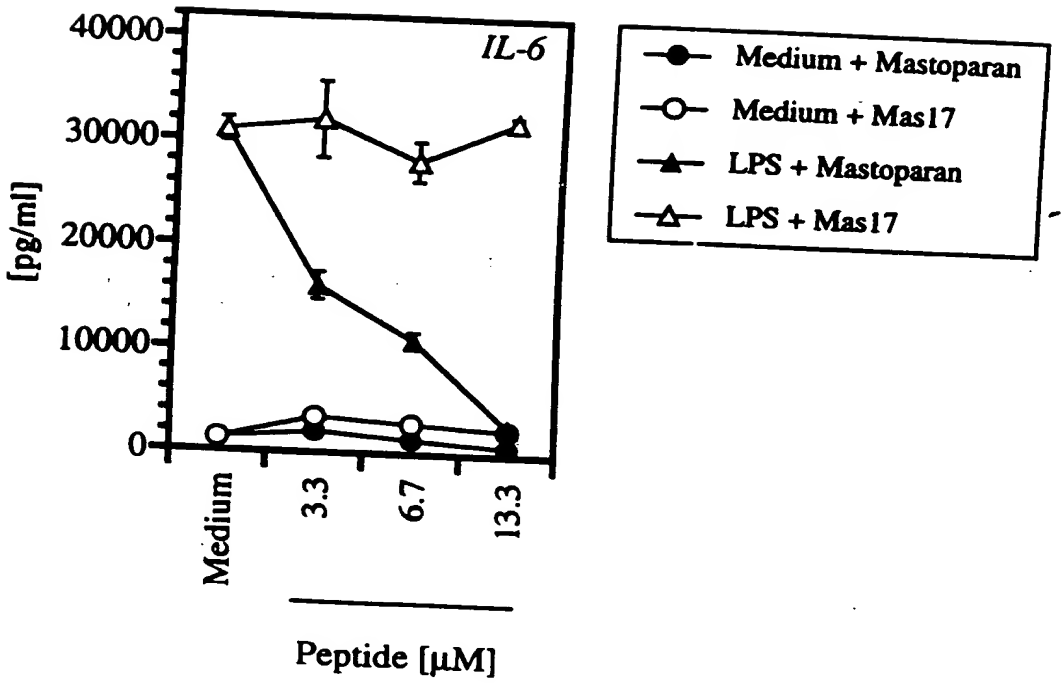

IL-6
40000
30000
20000
10000
0
[pg/ml]
Medium
3.3
6.7
13.3
Peptide [µM]
Medium + Mastoparan
Medium + Mas17
LPS + Mastoparan
LPS + Mas17

FIGURE 2

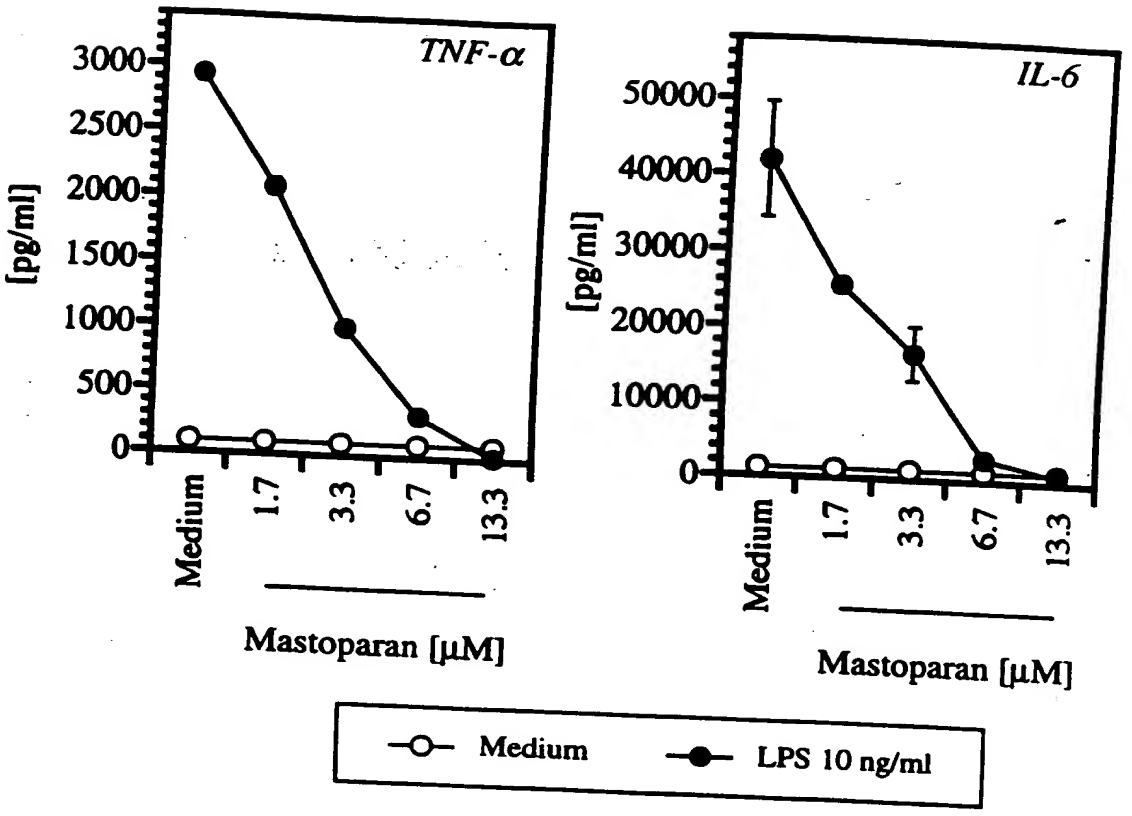
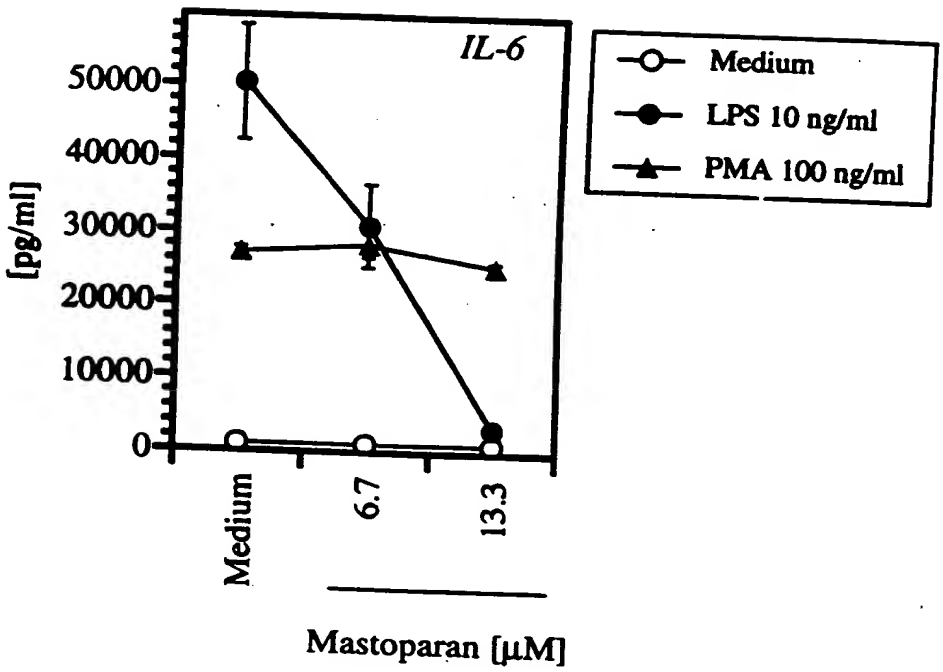

TNF-α
[pg/ml]
3000
2500
2000
1500
1000
500
0
Medium
1.7
3.3
6.7
13.3
Mastoparan [μM]
IL-6
[pg/ml]
50000
40000
30000
20000
10000
0
Medium
1.7
3.3
6.7
13.3
Mastoparan [μM]
Medium
LPS 10 ng/ml

FIGURE 3A


IL-6
Medium
LPS 10 ng/ml
PMA 100 ng/ml
[pg/ml]
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40000
30000
20000
10000
0
Medium
6.7
13.3
Mastoparan [µM]

FIGURE 3B

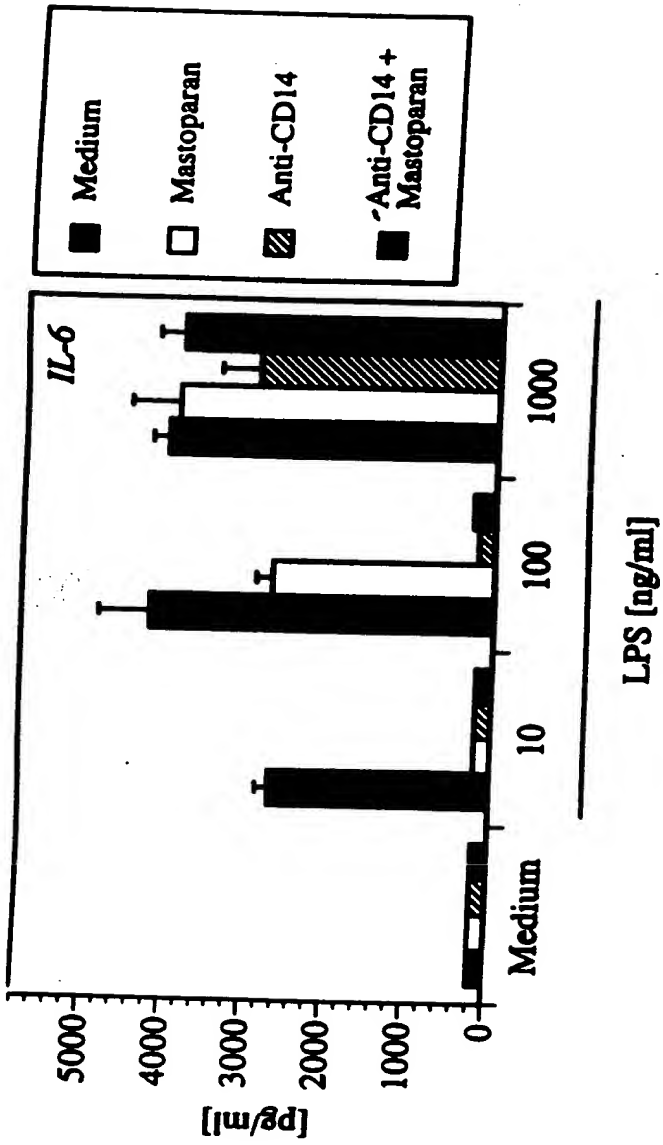
Medium
Mastoparan
Anti-CD14
Anti-CD14 + Mastoparan
IL-6
[pg/ml]
5000
4000
3000
2000
1000
0
Medium
10
100
1000
LPS [ng/ml]

FIGURE 4

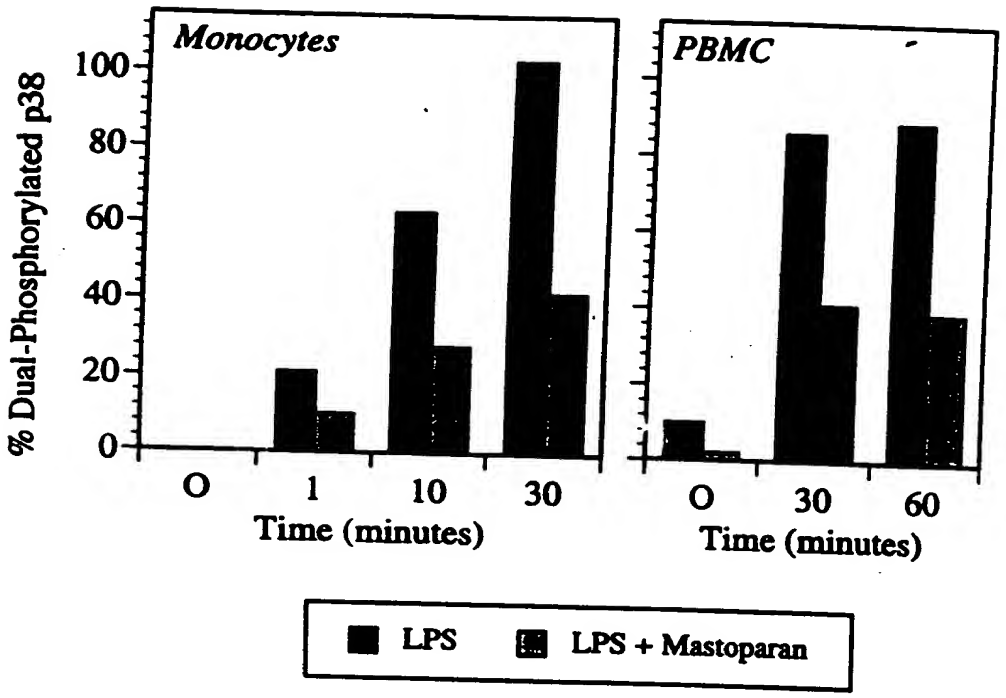
Monocytes
PBMC
% Dual-Phosphorylated p38
100
80
60
40
20
0
O
1
10
30
Time (minutes)
O
30
60
Time (minutes)
LPS
LPS + Mastoparan

FIGURE 5A

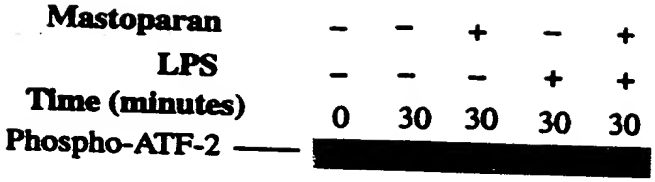

FIGURE 5B

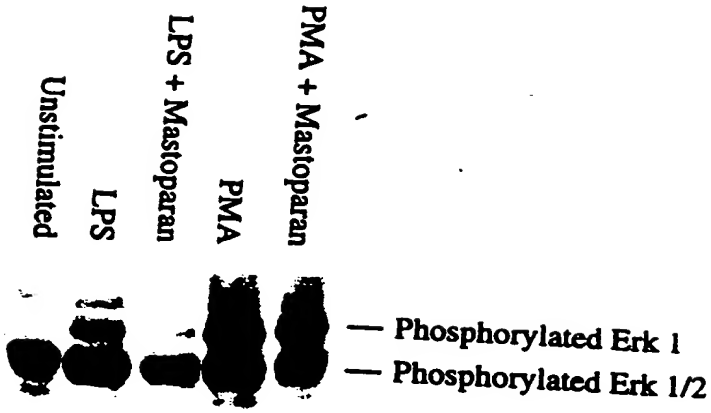
Unstimulated
LPS
LPS + Mastoparan
PMA
PMA + Mastoparan
Phosphorylated Erk 1
Phosphorylated Erk 1/2

FIGURE 6

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Applicant FINBERG, Robert, W. et al	

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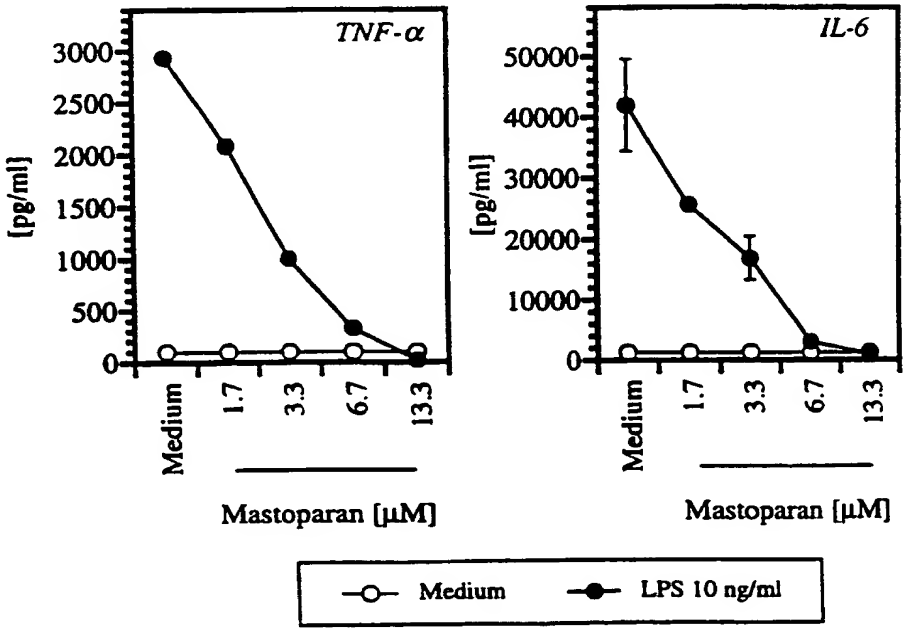

(57) Abstract

The present invention provides for the use of G protein binding agents for prophylactic and/or therapeutic treatment of septic shock. The present invention provides methods of using agents which bind G protein to treat a subject having or susceptible to septic shock. The present invention further pertains to compositi ns for treating a subject for septic shock. The composition includes an effective amount of a G protein binding agent such as mastoparan and, optionally, an antibiotic and a pharmaceutically acceptable carrier. Other aspects of the invention include packaged agents which bind G proteins for treating septic shock.

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THE USE OF AGENTS WHICH BIND G PROTEINS FOR TREATING SEPTIC SHOCK

Background of the Invention

Septic shock (also known as sepsis) causes more than 150,000 deaths annually in the United States. Sepsis is defined as a clinical disorder whose symptoms may include well defined abnormalities in body temperature, heart rate, breathing rate, white blood cell count, hypotension, organ perfusion abnormalities, and multiple organ dysfunction. There are several causes of sepsis including bacterial (either gram negative or gram positive), fungal and viral infections, as well as non-infective stimuli such as multiple trauma, severe burns, organ transplantation and pancreatitis.

Septic patients usually die as a result of poor tissue perfusion and injury followed by multiple organ failure. It is well recognized that many of the responses that occur during septic shock are initiated by bacterial endotoxin, a glycolipid antigen present on the surface of gram negative bacteria. This endotoxin (also referred to herein as lipopolysacchride or LPS) is released upon the death or multiplication of the bacteria and is known to activate monocytes/macrophages or endothelial cells causing them to produce various mediatior molecules such as toxic oxygen radicals, hydrogen peroxide, tumor neurosis factor-alpha (TNFα), and interleukin (IL-1, IL-6, and IL-8). Theses cellular and humoral inflammatory mediators evoke septic shock with symptoms ranging from chills and fever to circulatory failure, multiorgan failure, and death.

The impact of sepsis is particularly devastating to patients with compromised cardiac and hepatic function and to immunocompromised patients. Patients at high risk are elderly, chemothearpy patients and those requiring surgery or invasive instrumentation. The current therapy of antibiotics and hemodynamic support has not proven to be successful. An improved method for treating or preventing septic shock would be of great value.

The major LPS receptor for monocytes/macrophages is the glycosylphosphatidylinositol (GPI) anchored glycoprotein CD14. It is the interaction of LPS with the LPS receptor CD14 that initiates the cascade of signaling events that cause cytokine gene transcription. The precise mechanism through which LPS interacts with CD14 is unknown. Much of the controversy regarding the role of CD14 in LPS-induced signal transduction and cytokine production stems from the fact that CD14 is attached to the cell membrane by a glycosylphosphatidylinositol (GPI)-anchor and contains neither transmembrane nor cytoplasmic amino acid sequences. As such, CD14 cannot interact with signal transduction molecules in the same way as transmembrane receptors. Recently, it has been demonstrated that GPI-anchored proteins expressed on many cell

types can physically interact with lipid-linked signal transduction molecules, but the functional consequences of these interactions remain unresolved (Stefanova et al., *Science*, 254: 1016-1018, 1991; Shenoy-Scaria et al., *Mol Cell Biol.*, 13: 6385-6392, 1993; Solomon et al., *Proc. Nat. Acad. Sci.*, 93: 6053-6058, 1996).

Although the precise mechanism through which LPS binding to CD14 leads to cell activation is not known, it has been demonstrated that this interaction is enhanced by the serum factor LPS-binding protein (LBP) (Shumann et al., *Science*, 249: 1429-1432, 1990; Hailman et al., *J. Exp. Med.*, 179: 269-277, 1994). The interaction of LPS/LBP with CD14 causes the exchange of LPS with lipids in target membranes (Wurfel et al., *J. Exp. Med.*, 181: 1743-1754, 1997; Yu et al., *J. Clin. Med.*, 99: 315-324, 1997; Wurfel et al., *J. Immunol.*, 158: 3925-3934, 1997). It has been suggested that this lipid transfer is responsible for LPS-induced signal transduction. The rate of the exchange reaction depends on the lipid composition of the target membranes, which has led to speculation that CD14 functions only to direct LPS insertion into particular membrane domains (Hailman et al., *J. Exp. Med.*, 179: 269-277, 1994; Wurfel et al., *J. Immunol.*, 158: 3925-3934, 1997). While the mechanism that leads to LPS-induced signal transduction has not been demonstrated, it is known that monocyte activation by LPS leads to the phosphorylation of p38 mitogen activated protein kinase (MAPK), and production of inflammatory cytokines (i.e., TNF-α, IL-6) (Sweet, M.J. and Hume D.A., *J. Leuk. Biol.*, 60: 8-26, 1996).

Summary of the Invention

The present invention is based, at least in part, on the discovery that CD14 on monocytes/macrophages physically interacts with heterotrimeric G proteins and, in particular, that such G proteins specifically regulate LPS-induced mitogen activated protein (MAP) kinase activation and cytokine production in human monocytes/macrophages. This invention is further based on the discovery that agents which bind G proteins, such as G protein binding peptides, inhibit G protein signal transduction to thereby treat or prevent septic shock *in vivo*.

Accordingly, this invention provides compositions and methods for treating or preventing septic shock in a subject at risk of developing septic shock The method comprises administering an effective amount of an agent which binds G protein such that septic shock is treated or prevented in the subject. The agents which bind G protein are useful for both proplylactic and/or therapeutic treatments of septic shock.

The invention also pertains to compositions for treating or preventing septic shock in a subject which include an effective amount of an agent which binds G protein

and, optionally, an antibiotic. The composition can further include a pharmaceutically acceptable carrier.

The present invention also provides methods for using agents which bind G proteins in combination with other agents and/or treatment regimens (e.g., antibiotics, intravenous fluids, cardiovascular and respiratory support) to prophylactically and/or therapeutically treat a subject for septic shock. Other aspects of the invention include packaged agents which bind G proteins and instructions for using such agents for treatment of septic shock.

Brief Description of the Drawings

Figures 1A and 1B are gels depicting the association of CD14 with src kinases and heterotrimeric G proteins. Figure 1A shows that CD14 is associated with tyrosine phosphorylated proteins. Figure 1B shows that CD14 is associated with various src kinases and heterotrimeric G protein α subunits.

Figure 2 depicts the inhibition of IL-6 production from LPS-stimulated PMBCs by mastoparan. Figure 2 shows that mastoparan but not its inactive analogue (MAS-17) inhibits LPS-induced cytokine production.

Figures 3A and 3B depict the inhibition of cytokine production from LPS-stimulated monocytes by mastoparan. Figure 3A shows a dose-dependent inhibition of IL-6 and TNF-α production in human monocytes by mastoparan. Figure 3B shows inhibition of LPS-, but not PMA-, induced cytokine production from human monocytes by mastoparan. The experiments shown are representative of 6 different assays with similar results.

Figure 4 shows that mastoparan only inhibits CD14-dependent LPS-induced signal transduction in U373-CD14 tranfected cells.

Figures 5A and 5B depict the inhibition of phosphorylation of p38 MAP kinase in human monocytes and PMBC by mastoparan. Figure 5B shows that mastoparan reduces LPS-induced p38 MAP kinase activity.

Figure 6 is a gel depicting the effect of LPS and mastoparan on MAP kinase activation in human monocytes. Figure 6 shows specific inhibition of LPS-induced Erk kinase activation by mastoparan.

Detailed Description of the Invention

The present invention pertains to a method for treating or preventing septic shock in a subject by administering to the subject an effective amount of an agent which binds to G protein, such that septic shock in the subject is treated or prevented. Septic shock is commonly associated with bacterial infection in a surgical setting or with

immunocompromised subjects, and is typically characterized by abnormalities in body temperature, heart rate, blood pressure and breathing which can progress to spontaneous clotting in blood vessels, multiple organ failure and death. The present invention also pertains to a composition for treating or preventing septic shock in a subject which includes an effective amount of an agent which binds G protein to treat or prevent septic shock in the subject. The composition can further include an antibiotic and/or a pharmaceutically acceptable carrier.

The term "septic shock" or "sepsis" refers to a clinical disorder whose symptoms may include well defined abnormalities in body temperature, heart rate, breathing rate, white blood cell count, hypertension, organ perfusion abnormalities, and multiple organ dysfunction. It may be caused by bacterial (either gram negative or gram positive), fungal, viral or other infection, as well as by non-infective stimuli such as multiple trauma, severe burns, organ transplantation and pancreatitis. Septic shock is commonly caused by "gram-negative" endotoxin-producing aerobic rods --*Escherichia coli, Klebsiella pneumoniae, Proteus* species, *Pseudomonas aeruginosa* and *Salmonella*. Septic shock involved with gram negative bacteria is referred to as "endotoxic shock". A significant portion of the peripheral responses occurring during septic shock are initiated by endotoxin (also referred to herein as lipopolysaccharide or "LPS"), an outer-membrane component of gram-negative bacteria which is released upon the death or multiplication of the bacteria. The manner in which LPS evokes its effects is by binding to cells such as monocytes/macrophages or endothelial cells and triggering them to produce various mediators, such as oxygen radicals, hydrogen peroxide, tumor necrosis factor-alpha (TNF-α), and various interleukins (IL-1, IL-6, and IL-8). Gram-positive bacteria, particularly pneumococcal or streptococcal, may produce a similar clinical syndrome as endotoxic shock. Thus, as used herein, the term "endotoxic shock" refers to septic shock involved with gram negative and/or gram positive bacteria.

Whatever the offending agent, the septic shock affects the peripheral circulation of a subject. The affects of septic shock include direct toxic injury to the subject which may induce arteriolar vasodilation and pooling of blood and elevated capillary pressure leading to the escape of plasma water into the interstitial compartment, further impinging on the circulating blood volume of the subject. In addition, septic shock leads to an inflammatory-immune reaction which results in the release of vasodilators in the subject, such as histamine and complement fractions, further reducing the effective circulating volume. Platelet activation and the formation of thromboxane A_2 may add an element of platelet aggregation, with activation of the clotting sequence and the induction of disseminated intravascular coagulation in the subject. The overall effect of septic shock in the subject is a decrease in blood volume.

The language "treat for septic shock" is intended to cover therapeutic and/or prophylactic treatments. The agents which bind G proteins can be used to protect a subject from damage on injury caused by septic shock or can therapeutically treat a subject prior to the onset of septic shock. For therapeutic treatment, agents which bind G protein can treat or reduce the damage or injury caused by septic shock in a subject having one or more symptoms of septic shock. Symptoms of septic shock in a subject include tremors, fever, falling blood pressure, rapid breathing, rapid heart beat, and skin lesions. Within hours or days, these symptoms can progress to spontaneous clotting in blood vessels, severe hypotension and multiple organ failure. The term "treat" as used herein refers reducing or preventing at least one symptom associated with sepsis. The treatment of a subject need not be complete restoration to the subjects previous state, but rather can be an amount sufficient to prevent mortality of the subject.

For prophylactic treatments, agents which bind G proteins are administered prior to the onset of symptoms of septic shock to prevent or inhibit septic shock in subjects at risk of developing or susceptible to septic shock. Subjects at risk of developing septic shock include the elderly, immunocompromised patients (e.g., chemotherapeutic and HIV patients), patients requiring surgery or invasive instrumentation, and patients with compromised cardiac and hepatic function. Administering to a subject an agent which binds G protein in these types of settings can be used to decrease the susceptibility of these patients to septic shock.

The term "subject" is intended to include mammals having septic shock, including one or more of the symptoms related to sepsis. Examples of such subjects include humans, dogs, cats, pigs, cows, horses, rats, and mice.

The term "administering" is intended to include routes of administration which allow the agent to perform its intended function of treating or preventing septic shock by binding to G protein. Examples of routes of administration which can be used include parental injection (e.g., subcutaneous, intravenous, and intramuscular), intraperitoneal injection, oral, inhalation, and transdermal. The injection can be bolus injections or can be continuous infusion. Depending on the route of administration, the agent can be coated with or disposed in a selected material to protect it from natural conditions which may detrimentally effect its ability to perform its intended function. When the agent is a peptide, such as mastoparan or analog thereof, the peptide can be modified at one or more of its termini to protect the peptide from degradation. Methods of protecting peptides from degradation are disclosed in U.S Patent No. 5,589,568 which is incorporated herein by reference. The agent can be administered with other agents and/or with a pharmaceuticlly acceptable carrier. Further, the agent can be administered as a mixture of agents which bind G proteins, which also can be coadministered with a

pharmaceutically acceptable carrier. The agent can be administered prior to the onset of septic shock or after the onset of septic shock.

The language "pharmaceutically acceptable carrier" is intended to include substances capable of being coadministered with the G protein binding agent, and which allows the agent to perform its intended function of treating septic shock or preventing septic shock. An example of such a carrier is saline. Any other conventional carrier suitable for use with the G protein binding agent also fall within the scope of the present invention.

The language "effective amount" of an agent which binds G protein is that amount necessary or sufficient to treat or prevent septic shock, e.g. prevent at least one of the various symptoms of septic shock, or that amount necessary to reduce the pathogenesis of septic shock. The effective amount can vary depending on such factors as the size and weight of the subject, the type of illness, the severity of the symptoms, or the particular agent used. One of ordinary skill in the art would be able to study the aforementioned factors and make the determination regarding the effective amount of G protein binding agent without undue experimentation.

The regimen of administration can affect what constitutes an effective amount. G protein binding agents can be administered to the subject either prior to or after the onset of septic shock. Further, several divided dosages, as well as staggered dosages, can be administered daily or sequentially, or the dose can be continuously infused or can be a bolus injection. Further, the dosages of the G protein binding agent(s) can be proportionally increased or decrease as indicated by the exigencies of the therapeutic or prophylactic situation.

G proteins (guanine nucleotide binding regulatory proteins) are important to regulatory mechanisms operating in all human cells. Impairment of their function can perturb the cell's response to hormonal signals and adversely affect many intracellular metabolic pathways, thus contributing to the development and maintenance of a wide variety of disease states. When functioning normally, G proteins act as an integral part of the signal transducing mechanism by which extracellular hormones and neurotransmitters convey their signals through the plasma membrane of the cell and thus elicit appropriate intracellular responses. In its simplest terms, the signal transducing mechanism of G protein can be said to comprise three distinct components. A receptor protein with an extracellular binding site specific for a given agonist; a membrane-bound effector protein that when activated catalyzes the formation of facilitates the transport of an intracellular second messenger, an example is adenylate cyclase which produces cyclic AMP (cAMP); and a third protein which functions as a communicator between

these two. G proteins fulfill this vital role as communicator in the generation of intracellular responses to extracellular hormones and agonists.

G proteins are composed of three polypeptide subunits, namely G alpha (α), G beta (β), and G gamma (γ). The conformation of each subunit and their degree of association changes during the signal transducing mechanism. These changes are associated with the hydrolysis of the nucleotide GTP to form GDP and P sub i (GTPase activity). The binding sites for GTP, GDP and the GTPase catalytic site reside in the α subunit.

The G protein cycle which occurs each time a signal is conveyed across the membrane can be summarized as follows: 1) in an unstimulated cell the G proteins are found in the resting state in which α, β and γ are complexed together and GDP is bound to G α, 2) the binding of an appropriate hormone or agonist to the receptor changes its conformation and causes it to activate the G protein by displacing GDP and allowing GTP to bind (this is the rate-limiting step of the G protein cycle), 4) when GTP is bound to G α it may dissociate from the β and γ complex and is able to bind to, and activate, adenylate cyclase which releases cAMP into the cytoplasm, 5) GTP is then hydrolysed to GDP and the cycle is complete.

A further attribute inherent in this system is that it allows several different receptors to interact with a signal-generating enzyme. Some act in such a way to activate the enzyme and some to inhibit it. This involves distinct alpha subunits G sub s alpha (stimulatory) and G sub i alpha (inhibitory) that combine with the same beta gamma complex to form stimulatory or inhibitory G proteins. An example of a receptor that interacts with G sub i to lower the concentration of cAMP is the alpha sub 2-adrenergic receptor. The integration of the signals from G sub s and G sub i is one of the ways in which the level of cAMP in the cell can be modulated in response to several different extracellular agonists. The present invention is based on, at least in part, the demonstration of both a physical and functional coupling of CD14 to G proteins and the involvement of G proteins in the regulation of LPS-induced signal transduction.

The term "agent which binds to G protein" or "G protein binding agent" refers to an agent which binds to G protein and inhibits G protein signal transduction, such as a small molecule, compound, drug, polypeptide, or peptide. Preferred G protein binding agents bind a G α subunit. Particularly preferred agents are cell permeable agents. A particularly preferred G protein binding agent for treating bacterial sepsis is a peptide. Examples of such peptides include mastoparan and analogs thereof. Mastoparan is a permeable, amphiphilic peptide that binds Gi and Go heterotrimeric G proteins (Higashijima et al., *J. Biol. Chem.*, 263: 6491-6494, 1988; Higashijima et al., *J. Biol. Chem.*, 265: 14176-14186, 1990; Higashijima et al., *J. Biol. Chem.*, 265: 14176-14186,

1991). Mastoparan is the prototype of a family of peptide toxins, collectively known as mastoparans, that form amphiphilic alpha helices. Mastoparan has been shown to stimulate guanine nucleotide exchange by G proteins in a manner similar to that of G protein-coupled receptors. The mastoparans of the present invention can be naturally occurring mastoparans, or analogs thereof, which are known in the art and are described in U.S. Patent 5,589,568, incorporated herein by reference. Mastoparan can be synthesized and purified as described by Saito (*Chem. Pharm. Bull.*, 32: 2187-2193, 1984) incorporated herein by reference, or alternately can be purchased from Sigma (St. Louis, MO). The peptides of the present invention can be prepared by standard peptide synthesis technology (e.g., Merrifield, *J. Am. Chem. Soc.*, 88: 2149-2154, 1963; Houghton et al., *Int. J. Pept. Protein Res.*, 16: 311-320, 1980; Eler, *J. Biochem.*, 145: 157-162, 1984; *PNAS USA*, 82: 5131-5135, 1988), for example, by using a solid phase peptide synthesizer and purified by reverse phase HPLC. Such methods of peptide synthesis and purification are known in the art.

Agents which bind G proteins and inhibit G protein signal transduction can be identified by use of one or more assays known to those skilled in the art (for a review see Kaziro, Y. et al. (1991) *Ann. Rev. Biochem.*, 60: 349-400; and Neer, E. J., (1995) *Cell* 80: 249-257) or described herein. For example, G protein binding agents which inhibit G protein signal transduction can be identified by their ability to inhibit the production of cytokines (such as IL-6 and TNF-α) when cells (such as monocytes or macrophages) are contacted with the agent and a G protein stimulator, such as LPS. Alternatively, an agent which binds G protein can be identified by its ability to inhibit MAP kinase activation, under conditions as described herein. The *in vivo* efficacy of an agent which binds G protein and inhibits G protein signal transduction can be determined by the ability of such an agent to protect rats from LPS-induced lethal endotoxic shock as described herein.

The present invention also pertains to compositions and methods for treating a subject having septic shock or susceptible to septic shock. The composition contains an effective amount of an agent which binds G protein and a pharmaceutically acceptable treatment solution.

The present invention further pertains to the use of agents which bind G proteins together with an antibiotic for prophylactic and/or therapeutic treatments of septic shock. The invention also pertains to compositions for treating a subject for septic shock which include an effective amount of the antibiotic and an agent which binds G protein in a pharmaceutically acceptable carrier.

The present invention further pertains to the use of G protein binding agents in conjunction with other agents or regimens for therapy or prophylactic treatment of septic

shock. Some specific examples of other agents or regimens which can be administered to the subject to treat septic shock include antibiotics, intravenous fluids and cardiovascular and respiratory support.

The invention is further illustrated by the following examples which in no way should be construed as being further limiting. The contents of all references, pending patent applications, published patent applications, and issued patents cited throughout this application are hereby incorporated by reference. It should be understood that the animal models used throughout the examples are accepted animal models and that the demonstration of efficacy in these animal models is predictive of efficacy in humans.

EXAMPLES

The following materials and methods were used throughout the examples.

Isolation of human PBMC and monocytes.

Freshly isolated human peripheral blood mononuclear cells (PBMC) and monocytes were obtained from leukopaks (discarded leukocyte from platelet donations). The cells were fractionated on FICOLL-HYPAQUE™ gradients, washed, treated with tris-buffered NH_4Cl to eliminate RBCs and washed to obtain PMBCs. Monocytes were obtained by depleting the PBMCs of T cells and NK cells by negative selection asking standard techniques. T cells and NK cells were removed by treatment with anti-CD3 and anti-CD2 monoclonal antibodies followed by goat anti-mouse Ig conjugated magnetic beads at a 10:1 bead:cell ratio. The monocyte preparations were at least 80-85% monocytes, as determined by anti-CD14 staining and forward and slide light scatter analysis using a FACScan (Becton-Dickenson, Elmhurst, IL). Less than 2% of the contaminating cells in the monocyte preparation were T cells and no NK cells could be detected. Monocytes were maintained in Ham's F-12 10% FCS, L-Glutamine and penacillin/streptomyocin at 37°C in 5% CO_2.

Cell Lines

U373 cell CD14 transfectants (U373-CD14) were maintained in EMEM supplemented with 10% FCS, L-Glutamine and penicillin/streptomyocin at 37°C in 5% CO_2.

Immunoprecipitation, *in vitro* kinase assay, and reimmun precipitation.

Assays were performed as described in Solomon et al., *Proc. Nat. Acad. Sci, 93*:6053-6058 (1996). Briefly, cells were washed 3 times in cold buffered saline and were lysed on ice for 30 minutes in lysis buffer [0.5% NP40; 300 mM NaCl; 50 mM Tris pH 7.6; 0.15 u/ml aprotinin; 10 mM Iodoacetimide; 5 mM EDTA; 1 mM Na_3VO_4 10 μg/ml leupeptin; 1 mM PMSF]. Insoluble debris was removed by microcentrifugation and the lysates were precleared with 100 μl (10% w/v) rabbit anti-mouse coated protein A sephrose beads (1 mg/ml) followed by 200 μl (10% w/v) protein A sephrose beads. The lysates were then incubated for two hours at 4°C with monoclonal antibodies previously bound to protein A sephrose beads. After 2 hours, the beads were washed 4 times in lysis buffer and once in Kinase buffer [25 mM hepes, 1 mM $MnCl_2$ and 100 μM Na_3VO_4] and the immunoprecipitates were then resuspended in 50 μl kinase buffer with 20μCi[γ^{32}P]ATP (New England Nuclear, Boston, MA) and incubated for 15 minutes at room temperature. The samples were washed 4 times in lysis buffer with 15 mM EDTA. Samples were then eluted in 0.5% SDS at 70°C for 3 minutes or boiled in 1% SDS for 5 minutes and diluted 10 fold with cold lysis buffer. Samples were analyzed by SDS-PAGE or reimmunoprecipitated with various monoclonal or polyclonal antisera (e.g., rabbit) prior to SDS-PAGE analysis. Reimmunoprecipitated samples were boiled in reducing Laemmli sample buffer, and subjected to electrophoresis through a 10% SDS-PAGE gel.

Cytokine production

Freshly isolated human monocytes, human PBMC and U373-CD14 cells were incubated with 10 ng/ml LPS (*E coli* 0111:B4, Sigma, St. Louis, MO), 100 ng/ml PMA or were untreated in supplemented RPMI [10% FCS; 2 mM L-glutamine and penicillin/streptomycin] with or without various concentrations of mastoparan for 18 hours at 37°C in 24 well tissue culture dishes. IL-6 and TNF-α levels were determined by ELISA (Endogen Inc., Boston, MA) of supernatants harvested at 4 hours (for TNF-α) and at 18 hours (for IL-6) after LPS stimulation.

Immunoblotting

For detection of p38, phosphorylated p38 and phosphorylated Erk kinases in monocyte lysates, monocytes were subjected to various treatments and lysed in boiling reducing Laemmli sample buffer. The lysates were subjected to electrophoresis through a 10% SDS PAGE gel and were then transferred to nitrocellulose (NC). After washing twice with TBS-Twwen 20(0.1%) the NC was placed in a solution of Ponceau S dye to ensure equal loading and left in blocking buffer [1x TBS; 0.1% TBS; 5% milk] for 1

hour. After blocking and washing, the NC was incubated with anti-p38 N + C terminus (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 2-3 hours or with anti-phosphorylated p38 and Erk antibodies (New England Biolabs, Inc., Beverly, MA) for 18 hours. Membranes were washed three times in TBS-Tween 20 and were incubated for 30 minutes with horseradish peroxidase conjugated donkey-α-rabbit antibody in blocking buffer (Amersham Corp., Arlington Heights, IL). Membranes were washed an additional six times [3 x TBS-Tween-20; 3 x TBS], and were developed by exposure to ECL chemicals (Amersham corp., Arlington Heights, IL) and visualized by exposure to film.

For detection of p38 in nuclear extracts, cells after a variety of treatments were subject to lysis and nuclear extraction as described in Lerder et al., *J. Immunol. 152*:77-86 (1994). The nuclear extracts were subjected to protein quantitation by Micro BCA assay (Pierce, Rockford, IL) using a BSA standard. The details of the blotting procedure were the same as above except that 5-10 ng of extract was used.

Lethal endotoxin shock

Wistar rats (200 g) were obtained from Charles River Laboratories. Rats were treated with 3mg/kg mastoparan by intravenous injection in the tail vein, immediately followed by 15 mg/kg lead acetate and 5 μg/kg LPS 0111:B4 intravenously. Mortality was assessed up to 96 hours following LPS treatment. Mortality frequency was compared by Fisher exact test and statistical analysis was performed using Yates corrected Chi square test..

Example 1. Association of CD14 with G Proteins Following LPS Stimulation

To elucidate the mechanism of LPS-induced signal transduction mediated through CD14, CD14 was immunoprecipitated from freshly isolated human monocytes and *in vitro* kinase assays performed to asses the association of CD14 with phosphorylated proteins.

Results from these assays revealed the presence of multiple tyrosine phosphorylated species which coimmunoprecipitated with CD14 (See Figure 1A). Reimmunoprecipitation of the product of these assays with an anti-phosphotyrosine specific antibody indicated that all the major phosphorylated species were tyrosine phosphorylated. Immunoprecipitation of the products of the *in vitro* kinase assay with heterosera and various monoclonal antibodies recognizing src family tyrosine kinases indicated that in human monocytes fyn, lyn and fgr src family kinases were all present in substantial quantities and a small amount of hck was observed on overexposed

autoradiographs (Figure 1B). There was no evidence for lck in the immunoprecipitates from human monocytes.

In vitro kinase assays of immunoprecipitated CD14 also revealed the presence of a 40 kD tyrosine phosphorylated species (Figures 1A and 1B). This protein could be immunoprecipitated from the products of CD14 *in vitro* kinase assays with a pan-anti-G protein antiserum which recognizes the GTP binding site of a variety of G proteins, as described in Solomon et al., *Proc. Nat. Acad. Sci,* 93: 6065-6058 (1996), indicating that this species was a G protein. Immunoprecipitation of the products of the CD14 *in vitro* kinase assays with antisera specific for α subunits of the heterotrimeric G proteins indicated that this 40 kD protein consisted of a combination of heterometrimeric G protein α subunits of a small amount of Gi α1 and larger amounts of Gi α2, Gi α3 and Go α. In contrast, Gs α was not found in association with CD14 from the CD14 *in vitro* kinase assay. Similar patterns of heterometrimeric G proteins and src family kinases were found to coimmunoprecipitate with CD14 from CHO- and U373-CD14 transfectants and are similar to the patterns of phosphoproteins associated with other GPI-anchored proteins.

In addition, there was no evidence for the β or γ subunits of heterotrimeric G proteins in these immunoprecipitates. This may be due to the documented dissociation of these subunits from the α subunit during detergent lysis (Chang et al., *J. Cell Biol.*, 126: 127-138, 1994) or simply be an indication that these subunits are not phosphorylated in the *in vitro* kinase reactions.

Thus, the fact that CD14 immunoprecipitates contained Gi and Go heterodimer G proteins suggests that G proteins may be involved in LPS-induced signaling.

Example 2. *In Vitro* Effect of Mastoparan on G Protein Signal Transduction

To investigate the functional consequences of the G protein/CD14 association, human cells (i.e., monocytes and PBMCs) and U373 cell transfectants expressing CD14 were treated with mastoparan (a cell permeable, amphiphilic peptide that binds Gi and Go heterotrimeric G proteins) and LPS to determine the effect on LPS-induced cytokine production.

Initially, the effect of mastoparan (QCB, Hopkinton, MA) and its inactive analogue, MAS-17 (QBC) on cytokine production from human PBMCs were tested. Freshly isolated PBMCs were treated with LPS and/or peptides (mastoparan or the MAS-17 control peptide) and IL-6 levels were measured in the tissue culture supernatants of the cells (Figure 2). PBMCs produced IL-6 in response to LPS, while neither mastoparan nor MAS-17 stimulated IL-6 production from the PBMCs. Mastoparan was a potent inhibitor of LPS-induced IL-6 production from the LPS

stimulated PBMCs, while MAS-17 had no effect on cytokine production. The effect of mastoparan on cytokine production was dose-dependent, and at a concentration of 13.3 μM mastoparan totally ablated LPS-induced IL-6 production from the PBMCs.

In order to determine if the effect of mastoparan on LPS-induced cytokine production was due to a direct effect on monocytes, a highly enriched monocyte population was tested for the effect of LPS and mastoparan on cytokine production from these cells. Freshly isolated human monocytes were treated with mastoparan and LPS, after which IL-6 and TFN cytokine levels were measured in the tissue culture supernatants of the cells (Figure 3A). Untreated monocytes did not produce detectable levels of cytokines, verifying that the isolation procedure had not activated these cells. LPS caused a dose-dependent stimulation of cytokines from isolated monocytes, whereas mastoparan induced neither IL-6 nor TNF production from these cells. When mastoparan was used in conjunction with LPS, cytokine production was diminished. Concentrations of mastoparan as low as 1.67 μM caused dramatic reductions in both IL-6 and TNF production in monocytes stimulated with LPS. Mastoparan at 13.34 μM concentrations totally ablated LPS-induced cytokine production from these cells. Mastoparan had little effect on cytokine production from PMA stimulated cells (Figure 3B), indicating the specificity of mastoparan action, and lack of mastoparan toxicity. Mastoparan had no effect on cell viability as measured by trypan blue uptake even after 36 hours of continuous mastoparan incubation.

In addition, the effect of mastoparan on LSP-induced cytokine production from an LPS responsive, CD14 transfected cell line was determined. LPS treatment of U373-CD14 transfectants induced an LPS dose-dependent production of IL-6 (Figure 4). At low concentrations of LPS (10 ng/ml-100 ng/ml) LPS-induced IL-6 responses were completely inhibited by treatment of the U373-CD14 cells with an anti-CD14 monoclonal antibody. IL-6 production induced by 10 ng/ml LPS was also ablated by treatment of the cells with mastoparan, while at 100 ng/ml of LPS, mastoparan reduced IL-6 levels by approximately one third. At high concentrations of LPS (1 μg/ml) the IL-6 responses of these cells were not inhibited by treatment with the anti-CD14 monoclonal antibody. Thus, at high concentrations of LPS, U373 cells exhibit CD14-independent LPS induced cytokine responses. At LPS concentrations of 1 μg/ml, mastoparan was ineffective at reducing cytokine responses from these cells. Thus, CD14-independent LPS signals were not inhibited by mastoparan.

Thus, pharmacologic targeting of the same heterotrimeric G proteins which are associated with CD14 had a substantial and specific impact on LPS induction of cytokine production.

LPS stimulation of cells through CD14 leads to the induction of a MAP kinase signaling pathway involving the p38 MAP kinase, which has been shown to be specifically induced by LPS (Figures 5A and 5B). Because mastoparan had profound effects on cytokine production and p38 MAP kinase is involved in LPS induced-signaling, the effect of mastoparan on MAP kinase activation was evaluated. For full activation, p38 MAP kinase requires phosphorylation on both threonine and tyrosine residues (Raingeaud et al., *J. Biol. Chem.*, 270: 7420-7426, 1995). Detection of dual-phosphorylated p38 MAP kinase by monoclonal antibodies specific for the dual-phosphorylated form of p38 was used as a measure of p38 activation. Consistent with the effect of mastoparan on LPS-induced cytokine production, mastoparan reduced the LPS-induced phosphorylation of p38 MAP kinase in both monocytes and PBMC (Figure 5A). It also inhibited LPS-induced nuclear translocation of p38.

Since it had been previously demonstrated that LPS induces phosphorylation of Erk 1 and 2 MAP kinases in transformed macrophage cell lines (Weinstein et al., *J. Immunol.*, 151: 3829-3838, 1993; Weinstein et al., *J. Biol. Chem.*, 267: 14955-14962, 1992), LPS-induced activation of ERK 1 and 2 and the effect of mastoparan on the activation of these kinases in normal human monocytes was determined (Figure 6). Only minimal phosphorylation of Erk 1 or 2 as seen in LPS-stimulated monocytes. The lack of substantial LPS-induced Erk kinase phosphorylation in freshly isolated monocytes is consistent with results obtained with other non-transformed human cells (Nick et al., *J. Immunol.*, 156: 4867-4875, 1996). In contrast to LPS, PMA induced substantial phosphorylation of Erk kinases. Interestingly, while mastoparan completely inhibited the Erk kinase activation induced by LPS, it had minimal effects on Erk kinase activation induced by PMA. This is consistent with the cytokine data, in so far as the effect of mastoparan was specific for the LPS signal transduction pathway and did not globally alter the ability to activate MAP kinases.

Example 3. *In Vivo* Effect of Mastoparan on G Protein Signal Transduction

The ability of mastoparan to inhibit cytokine production from human monocytes suggested that mastoparan may have efficacy in reducing LPS-induced pathology *in vivo*. To determine the effect of mastoparan *in vivo*, the effect of mastoparan on LPS-induced lethal endotoxic shock in rats was assessed.

Mastoparan did not have any toxic effects when used alone in the model system (data not shown). Mastoparan did significantly protect rats from LPS induced mortality (Table 1). These results demonstrate the importance of G protein-mediated events in endotoxic shock and that targeting heterotrimeric G proteins with pharmological agents which bind G proteins has therapeutic potential.

Table I

Group	Mortality		Total (%)
	14 hours	*24 hours*	
LPS	14/17	14/17	82.4%
LPS* + *Mastoparan	7/18	8/18	44.4%

Data were subjected to Yates corrected Chi square statistical analysis. The ability of mastoparan to protect rats from LPS-induced mortality was statistically significant $p<0.05$ at both 14 and 24 hours. The experiment was continued for 96 hours and the mortality remain unchanged from the 24 hour point.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments and methods described herein. Such equivalents are intended to be encompassed by the scope of the following claims.

What is claimed is

1. A method for treating or preventing septic shock in a subject comprising, administering to the subject an effective amount of an agent which binds G protein, such that septic shock in the subject is treated or prevented.

2. The method of claim 1, wherein the agent binds Gα subunit.

3. The method of claim 1, wherein the agent is a cell permeable agent.

4. The method of claim 3, wherein the agent is a peptide.

5. The method of claim 4, wherein the peptide is mastoparan or an analog thereof.

6. The method of claim 1, wherein the septic shock is endotoxic shock.

7. The method of claim 6, wherein the endotoxic shock is induced by gram negative bacteria.

8. The method of claim 1, wherein the endotoxic shock is induced by gram positive bacteria.

9. The method of claim 1, wherein the septic shock is LPS-induced shock.

10. The method of claim 1, further comprising administering an antibiotic to the subject.

11. A composition for treating or preventing septic shock in a subject comprising an effective amount of an agent which binds G protein to treat or prevent septic shock.

12. The composition of claim 11, wherein the agent binds Gα subunit.

13. The composition of claim 11, further comprising a pharmaceutically acceptable carrier.

14. The composition of claim 11, wherein the agent is a cell permeable agent.

15. The composition of claim 14, wherein the cell permeable agent is a peptide.

16. The composition of claim 15, wherein the peptide is mastoparan or an analog thereof.

17. The composition of claim 11, further comprising an antibiotic.

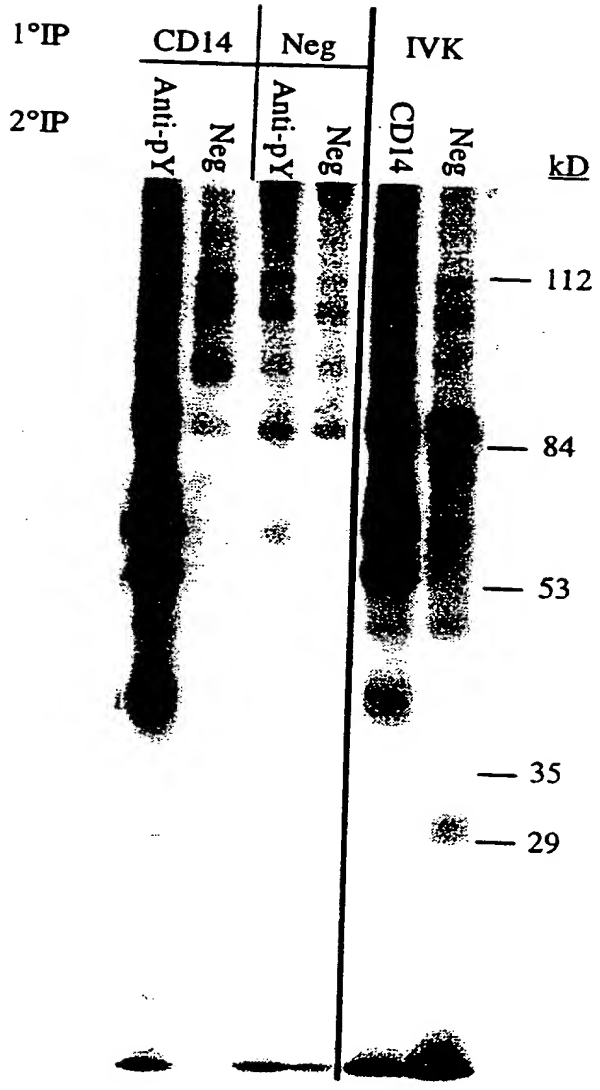
1°IP
CD14
Neg
IVK
2°IP
Anti-pY
Neg
Anti-pY
Neg
CD14
Neg
kD
112
84
53
35
29

FIGURE 1A

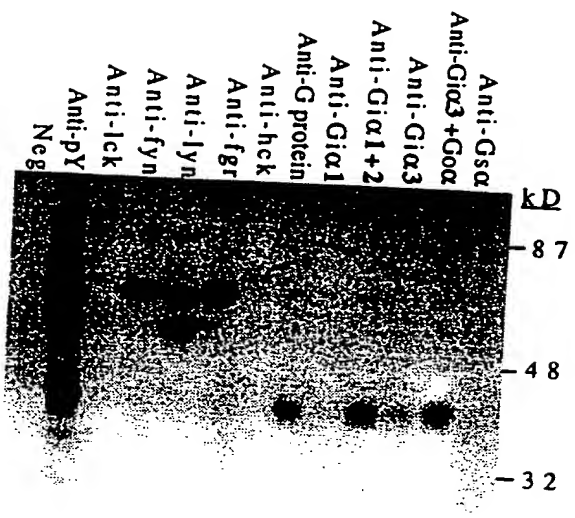
Neg
Anti-pY
Anti-lck
Anti-fyn
Anti-lyn
Anti-fgr
Anti-hck
Anti-G protein
Anti-Giα1
Anti-Giα1+2
Anti-Giα3
Anti-Giα3 +Goα
Anti-Gsα
kD
87
48
32

FIGURE1B

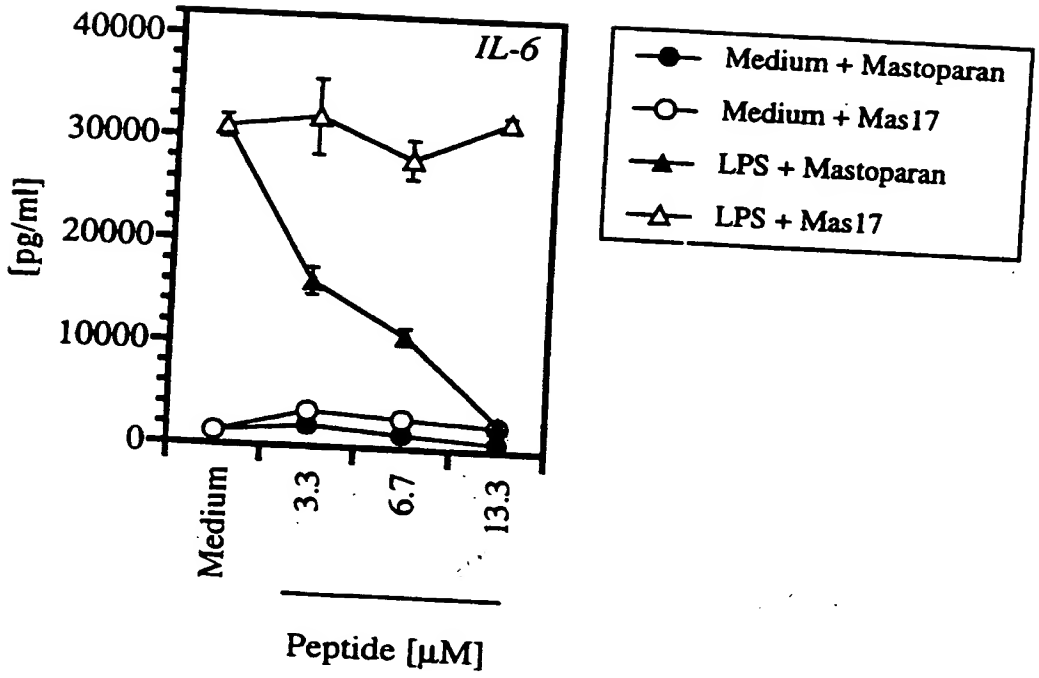
IL-6
40000
30000
20000
10000
0
[pg/ml]
Medium
3.3
6.7
13.3
Peptide [µM]
Medium + Mastoparan
Medium + Mas17
LPS + Mastoparan
LPS + Mas17

FIGURE 2

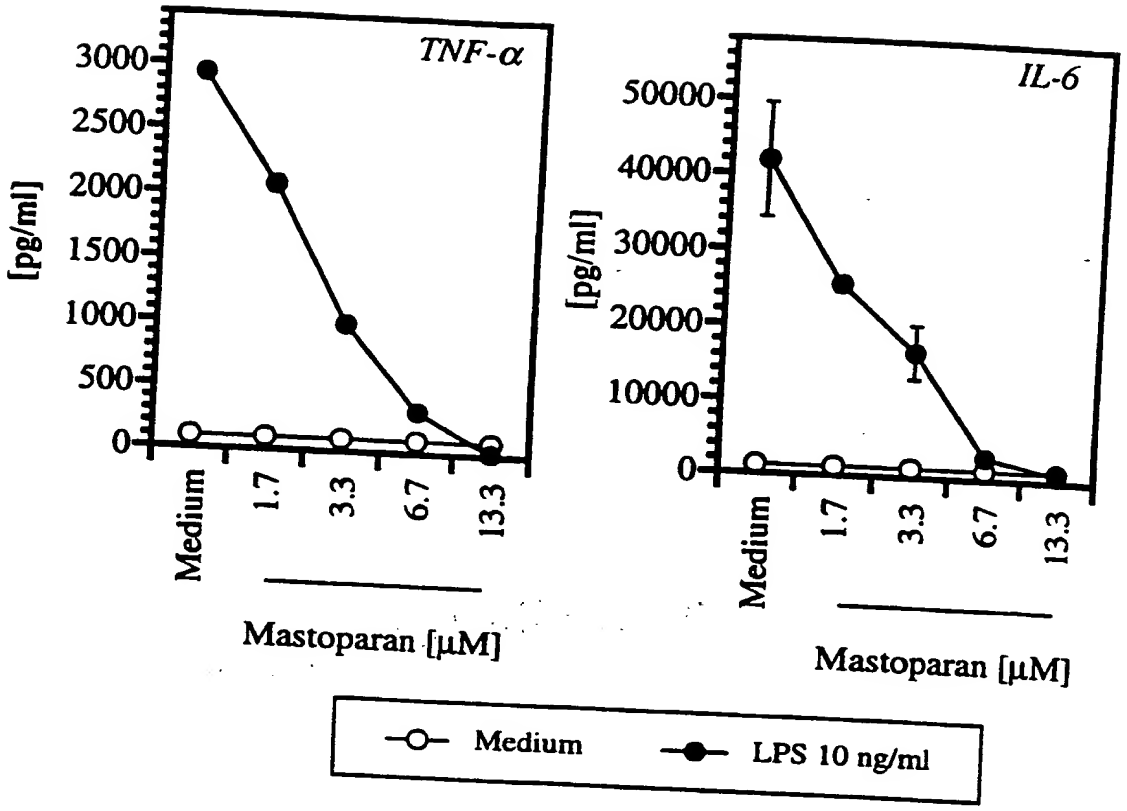
TNF-α
[pg/ml]
3000
2500
2000
1500
1000
500
0
Medium
1.7
3.3
6.7
13.3
Mastoparan [μM]
IL-6
[pg/ml]
50000
40000
30000
20000
10000
0
Medium
1.7
3.3
6.7
13.3
Mastoparan [μM]
Medium
LPS 10 ng/ml

FIGURE 3A

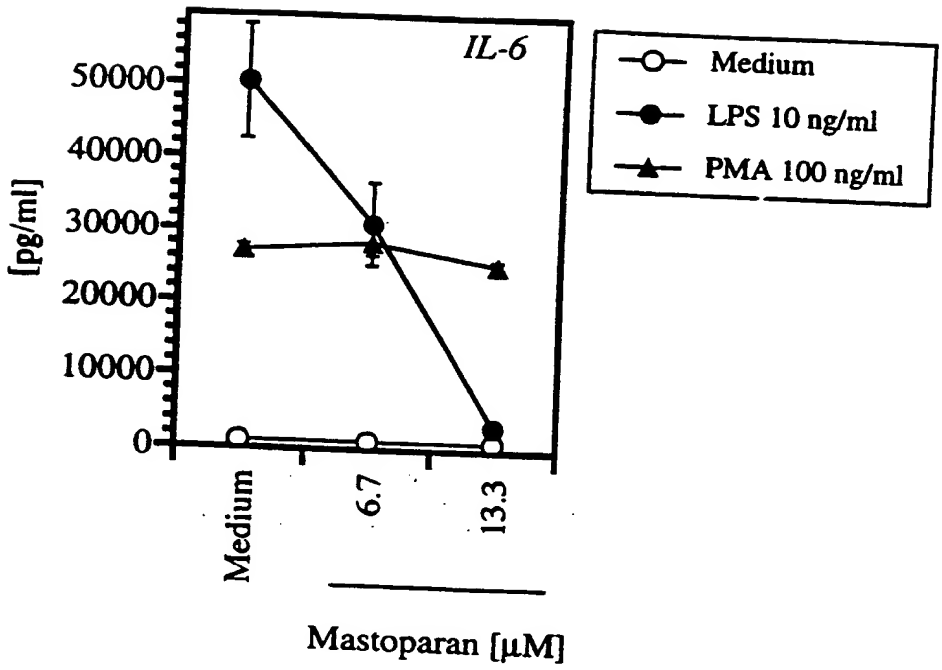
IL-6
50000
40000
30000
20000
10000
0
[pg/ml]
Medium
6.7
13.3
Mastoparan [µM]
Medium
LPS 10 ng/ml
PMA 100 ng/ml

FIGURE 3B

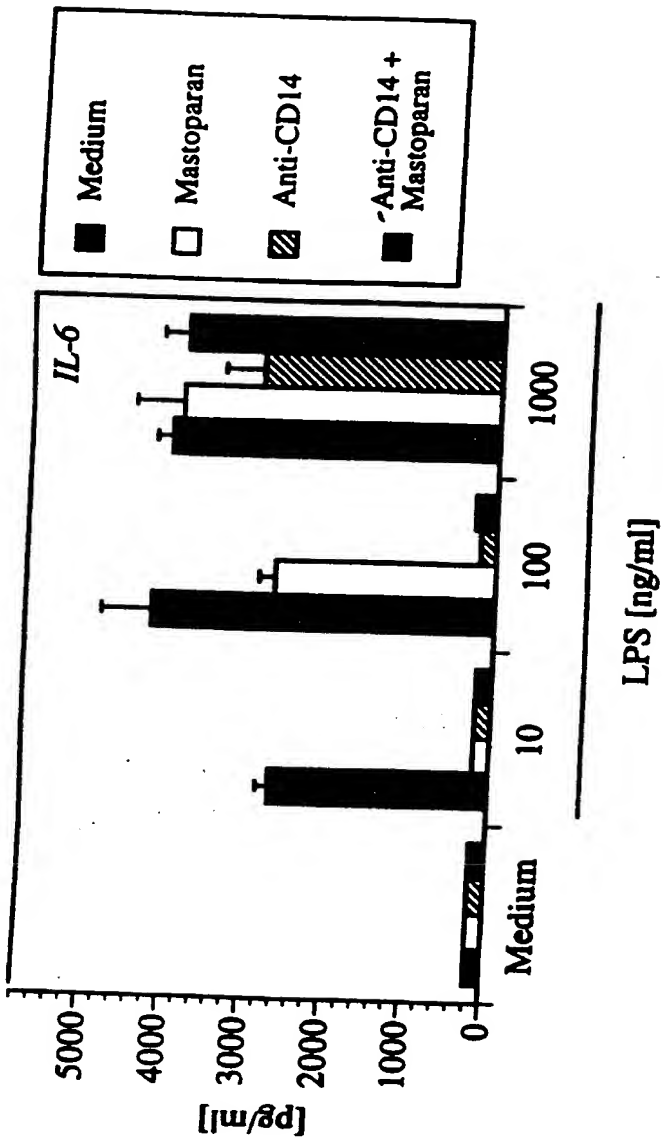
Medium
Mastoparan
Anti-CD14
Anti-CD14 + Mastoparan
IL-6
[pg/ml]
5000
4000
3000
2000
1000
0
Medium
10
100
1000
LPS [ng/ml]

FIGURE 4

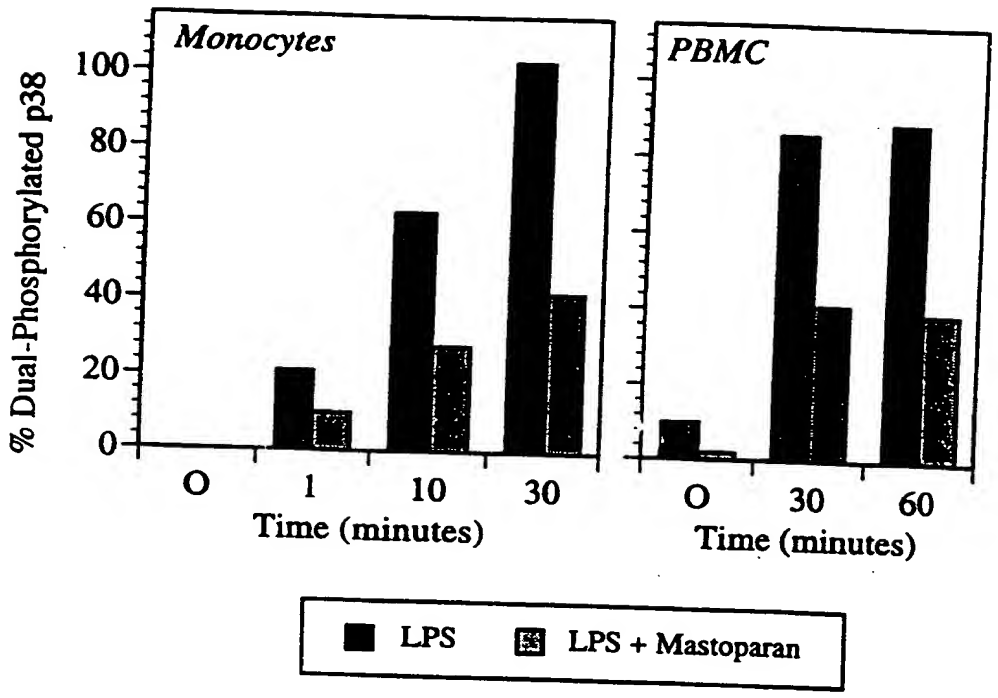
Monocytes
PBMC
% Dual-Phosphorylated p38
100
80
60
40
20
0
O
1
10
30
Time (minutes)
O
30
60
Time (minutes)
LPS
LPS + Mastoparan

FIGURE 5A

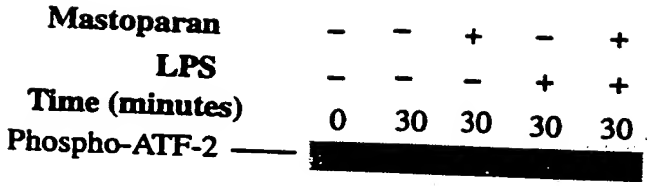

FIGURE 5B

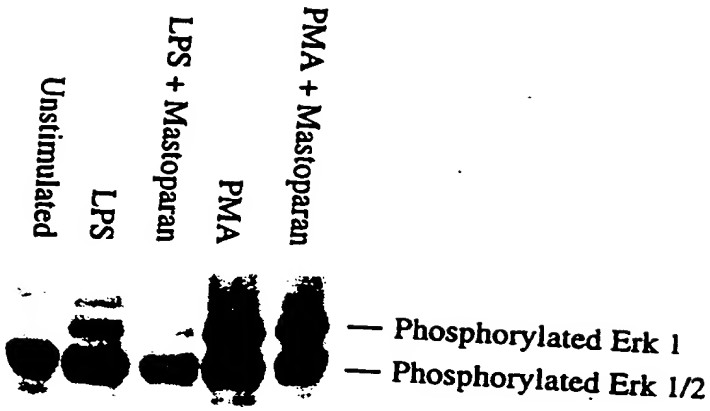
Unstimulated
LPS
LPS + Mastoparan
PMA
PMA + Mastoparan
Phosphorylated Erk 1
Phosphorylated Erk 1/2

FIGURE 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18432

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/02, 38/10
US CL :514/2, 14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 14

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG
search terms: mastoparan, G protein, septic shock, endotoxic shock, sepsis, antibiotic

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,492,898 A (BERTICS ET AL) 20 February 1996 (20/02/96), see column 2, lines 13-18, column 6, lines 28-34, column 9, line 67 - column 10, line 6.	1-3, 6-14
X	US 5,589,568 A (HIGASHIJIMA ET AL) 31 December 1996 (31/12/96), abstract, column 7, lines 1-20.	1-9, 11-16
X	CABEZA-ARVELAIZ et al. Cholera and Pertussis Exotoxins Protect Mice Against the Lethal Schwartzman Reaction and Antagonize the Effects of Lipopolysaccharide on Second Messenger Systems. Lymphokine Research. 1990, Volume 9, Number 2, pages 125-135, especially page 126, lines 12-17 and 24-31, page 128, Table 1, page 133, lines 6-17.	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 02 NOVEMBER 1998	Date of mailing of the international search report 19 NOV 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEFFREY E. RUSSEL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18432

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCTOR et al. Protection of mice from endotoxic death by 2-methylthio-ATP. Proceedings of the National Academy of Science USA. June 1994, Volume 91, pages 6017-6020, especially page 6020, column 1, Table 1 and first full paragraph.	1-3, 6-9, 11-14
X	SOLOMON et al. G proteins regulate LPS mediated cytokine release. Clinical Infectious Diseases. August 1997, Volume 25, Number 2, page 370, Abstract Number 83.	1-9, 11-16

Also of interest are the synthetic peptides disclosed in the following pending patent applications, peptides that have lytic activity with as few as 10-14 amino acid residues: McLaughlin *et al.*, "Amphipathic Peptides," United States patent 5,789,542, issued August 4, 1998; and Mark L. McLaughlin *et al.*, "Short Amphipathic Peptides with Activity against Bacteria and Intracellular Pathogens," United States patent application serial number 08/796,123, filed February 6, 1997.

Lytic peptides such as are known generally in the art may be used in practicing the present inventions. Selective toxicity to ligand-activated cells is desirable, especially when the ligand and peptide are administered separately. Selective toxicity is less important when the ligand and peptide are linked to one another, because in that case the peptide is effectively concentrated in the immediate vicinity of cells having receptors for the ligand.

Examples of such peptides are those designated D1A21 (SEQ. ID NO. 5), D2A21 (SEQ. ID NO. 6), D5C (SEQ. ID NO. 7), and D5C1 (SEQ. ID NO. 8). These peptides and other lytic peptides suitable for use in the present invention are disclosed in Jaynes, "Methods for the Design of Amphipathic Peptides Having Enhanced Biological Activities," United States provisional patent application serial number 60/027,628, filed October 4, 1996.

Miscellaneous

As used in the Claims, an "effective amount" of a composition is an amount that is sufficient to induce long-term contraception or sterility in a mammal. Where appropriate in context, an "effective amount" of GnRH is an amount sufficient to temporarily restore fertility in a mammal that has been made sterile by destruction of gonadotropic cells. As used in the Claims, the term "mammal" is intended to include both human and non-human mammals.

The complete disclosures of all references cited in this specification are hereby incorporated by reference; as are the full disclosures of United States provisional application 60/041,009, filed March 27, 1997; United States provisional application 60/057,456, filed September 3, 1997; United States non-provisional application 08/869,153, filed June 4, 1997; and international application PCT/US98/06114, filed March 27, 1998. In the event of an otherwise irreconcilable conflict, however, the present specification shall control.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Board of Supervisors of Louisiana State University and Agricultural and Mechanical College
Enright, Frederick M.
Jaynes, Jesse M.
Hansel, William
Elzer, Philip H.
Melrose, Patricia A.

(ii) TITLE OF INVENTION: Compositions and Methods for Contraception in or Sterilization of Mammals

(iii) NUMBER OF SEQUENCES: 13, 1 of which is deliberately blank

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: John H. Runnels
(B) STREET: P. O. Box 2471
(C) CITY: Baton Rouge
(D) STATE: LA
(E) COUNTRY: USA
(F) ZIP: 70821-2471

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 01-SEP-1998
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Runnels, John H.
(B) REGISTRATION NUMBER: 33,451
(C) REFERENCE/DOCKET NUMBER: 96A3.1-PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (225) 387-3221
(B) TELEFAX: (225) 346-8049

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..10
(D) OTHER INFORMATION: /note= "Xaa in position 1 denotes pyro-glutamic acid. This sequence is GnRH."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Xaa His Trp Ser Tyr Gly Leu Arg Pro Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..23
(D) OTHER INFORMATION: /note= "This sequence is hecate."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Phe Ala Leu Ala Leu Lys Ala Leu Lys Lys Ala Leu Lys Lys Leu Lys
1 5 10 15

Lys Ala Leu Lys Lys Ala Leu
20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..33
(D) OTHER INFORMATION: /note= "This sequence is a modified GnRH/hecate fusion peptide."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gln His Trp Ser Tyr Gly Leu Arg Pro Gly Phe Ala Leu Ala Leu Lys
1 5 10 15

Ala Leu Lys Lys Ala Leu Lys Lys Leu Lys Lys Ala Leu Lys Lys Ala
20 25 30

Leu

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..33
(D) OTHER INFORMATION: /note= "This sequence is a hecate/modified GnRH fusion peptide."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Phe Ala Leu Ala Leu Lys Ala Leu Lys Lys Ala Leu Lys Lys Leu Lys
1 5 10 15

Lys Ala Leu Lys Lys Ala Leu Gln His Trp Ser Tyr Gly Leu Arg Pro
20 25 30

Gly

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..23
(D) OTHER INFORMATION: /note= "This sequence is D1A21."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Phe Ala Phe Ala Phe Lys Ala Phe Lys Lys Ala Phe Lys Lys Phe Lys
1 5 10 15

Lys Ala Phe Lys Lys Ala Phe
20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..23
(D) OTHER INFORMATION: /note= "This sequence is D2A21."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Phe Ala Lys Lys Phe Ala Lys Lys Phe Lys Lys Phe Ala Lys Lys Phe
1 5 10 15

Ala Lys Phe Ala Phe Ala Phe
20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..27
(D) OTHER INFORMATION: /note= "This sequence is D5C."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys Arg Lys Arg Ala Val Lys Arg Val Gly Arg Arg Leu Lys Lys Leu
1 5 10 15

Ala Arg Lys Ile Ala Arg Leu Gly Val Ala Phe
20 25

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..37
(D) OTHER INFORMATION: /note= "This sequence is D5C1."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Lys Arg Lys Arg Ala Val Lys Arg Val Gly Arg Arg Leu Lys Lys Leu
1 5 10 15

Ala Arg Lys Ile Ala Arg Leu Gly Val Ala Lys Leu Ala Gly Leu Arg
20 25 30

Ala Val Leu Lys Phe
35

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..10
(D) OTHER INFORMATION: /note= "This sequence is a modified GnRH."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gln His Trp Ser Tyr Gly Leu Arg Pro Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

[SEQ ID NO 10 deliberately left blank]

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..15
(D) OTHER INFORMATION: /note= "This sequence is bLH."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ser Tyr Ala Val Ala Leu Ser Cys Gln Cys Ala Leu Cys Arg Arg
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..38
(D) OTHER INFORMATION: /note= "This sequence is a hecate-bLH fusion peptide."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Phe Ala Leu Ala Leu Lys Ala Leu Lys Lys Ala Leu Lys Lys Leu Lys
1 5 10 15

Lys Ala Leu Lys Lys Ala Leu Ser Tyr Ala Val Ala Leu Ser Cys Gln
20 25 30

Cys Ala Leu Cys Arg Arg
35

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..10
(D) OTHER INFORMATION: /note= "Xaa in position 1 denotes pyro-glutamic acid. Xaa in position 6 denotes D-lysine. This sequence is D-Lys-6 GnRH."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Xaa His Trp Ser Tyr Xaa Leu Arg Pro Gly
1 5 10

What is claimed:

1. A compound comprising: **(a)** a hormone domain selected from the group consisting of gonadotropin-releasing hormone, and analogues of gonadotropin-releasing hormone; and **(b)** a lytic peptide domain.

2. A compound as recited in Claim 1, wherein said hormone domain is bonded directly to said lytic peptide domain, without an intermediate linking domain joining said hormone domain to said lytic peptide domain.

3. A compound as recited in Claim 1, wherein said lytic peptide domain is selected from the group consisting of a cecropin peptide, a melittin peptide, a defensin peptide, a magainin peptide, a sarcotoxin peptide, and analogs of said peptides.

4. A compound as recited in Claim 1, wherein said lytic peptide domain comprises hecate.

5. A compound as recited in Claim 1, wherein said hormone domain comprises gonadotropin-releasing hormone.

6. A compound as recited in Claim 1, wherein said compound has the sequence SEQ. ID NO. 3.

7. A compound as recited in Claim 1, wherein said compound has the sequence SEQ. ID NO. 4.

8. A compound as recited in Claim 1, wherein said hormone domain, or said lytic peptide domain, or both comprise D-conformation amino acid residues.

9. A compound as recited in Claim 8, additionally comprising a carrier domain to facilitate uptake by the intestine when the compound is administered orally.

10. A compound as recited in Claim 9, wherein said carrier domain comprises a vitamin B_{12} domain.

11. A method for producing long-term contraception or sterility in a mammal, comprising administering to the mammal an effective amount of gonadotropin-releasing hormone and an effective amount of a lytic peptide.

12. A method as recited in Claim 11, wherein the lytic peptide is administered after the gonadotropin-releasing hormone is administered.

13. A method as recited in Claim 11, wherein the gonadotropin-releasing hormone, or the lytic peptide, or both comprise D-conformation amino acid residues.

14. A method as recited in Claim 13, wherein the compound containing D-conformation amino acid residues additionally comprises a carrier domain to facilitate uptake by the intestine when the compound is administered orally.

15. A method as recited in Claim 14, wherein the carrier domain comprises a vitamin B_{12} domain.

16. A method for producing long-term contraception or sterility in a mammal, comprising administering to the mammal a compound comprising a gonadotropin-releasing hormone domain, and a lytic peptide domain.

17. A method as recited in Claim 16, wherein the hormone domain is bonded directly to the lytic peptide domain, without an intermediate linking domain joining the hormone domain to the lytic peptide domain.

18. A method as recited in Claim 16, wherein the lytic peptide domain is selected from the group consisting of a cecropin peptide, a melittin peptide, a defensin peptide, a magainin peptide, a sarcotoxin peptide, and analogs of said peptides.

19. A method as recited in Claim 16, wherein the lytic peptide domain comprises hecate.

20. A method as recited in Claim 16, wherein the compound has the sequence SEQ. ID NO. 3.

21. A method as recited in Claim 16, wherein the compound has the sequence SEQ. ID NO. 4.

22. A method of temporarily restoring fertility in a mammal that had been made sterile by the selective destruction of gonadotropes in the pituitary, comprising administering to the mammal an effective amount of gonadotropin-releasing hormone.

23. A method as recited in Claim 22, wherein fertility is restored in a mammal that had previously been made sterile by administering to the mammal an effective amount of gonadotropin-releasing hormone and an effective amount of a lytic peptide.

24. A method as recited in Claim 22, wherein fertility is restored in a mammal that had previously been made sterile by administering to the mammal an effective amount of a compound comprising a gonadotropin-releasing hormone domain, and a lytic peptide domain.

25. A method as recited in Claim 11, wherein the mammal is a dog.

26. A method as recited in Claim 11, wherein the mammal is a cat.

27. A method as recited in Claim 11, wherein the mammal is a cow or bull.

28. A method as recited in Claim 11, wherein the mammal is a pig.

29. A method as recited in Claim 11, wherein the mammal is a horse.

30. A method as recited in Claim 11, wherein the mammal is a sheep.

31. A method as recited in Claim 11, wherein the mammal is a human.

32. A method as recited in Claim 16, wherein the mammal is a dog.

33. A method as recited in Claim 16, wherein the mammal is a cat.

34. A method as recited in Claim 16, wherein the mammal is a cow or bull.

35. A method as recited in Claim 16, wherein the mammal is a pig.

36. A method as recited in Claim 16, wherein the mammal is a horse.

37. A method as recited in Claim 16, wherein the mammal is a sheep.

38. A method as recited in Claim 16, wherein the mammal is a human.

39. A method for selectively killing gonadotrophic cells in the pituitary of a mammal, comprising administering to the mammal: **(a)** an effective amount of gonadotropin-releasing hormone, and **(b)** an effective amount of a lytic peptide.

40. A method for selectively killing gonadotrophic cells in the pituitary of a mammal, comprising administering to the mammal an effective amount of a compound comprising a gonadotropin-releasing hormone domain and a lytic peptide domain.

41. A method for selectively killing neurons having gonadotrophic receptors in a mammal, comprising administering to the mammal: **(a)** an effective amount of gonadotropin-releasing hormone, and **(b)** an effective amount of a lytic peptide.

42. A method for selectively killing neurons having gonadotrophic receptors in a mammal, comprising administering to the mammal an effective amount of a compound comprising a gonadotropin-releasing hormone domain and a lytic peptide domain.

43. A method as recited in Claim 11, wherein the mammal is sexually immature.

44. A method as recited in Claim 16, wherein the mammal is sexually immature.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18117

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/09; C07K 7/23
US CL : 514/2, 12, 841; 530/313, 324

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 12, 841; 530/313, 324

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,631,007 A (RYALS et al.) 20 May 1997, col. 21 to col. 22.	1-44

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18117

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN, EMBASE, WPIDS, MEDLINE, BIOSIS
search terms: gonadotropin releasing hormone, gnrh, grh, hecate, lytic, lysis, ligand, cytotoxin, amphipathic, peptide, target, hormone, steril/, contraception

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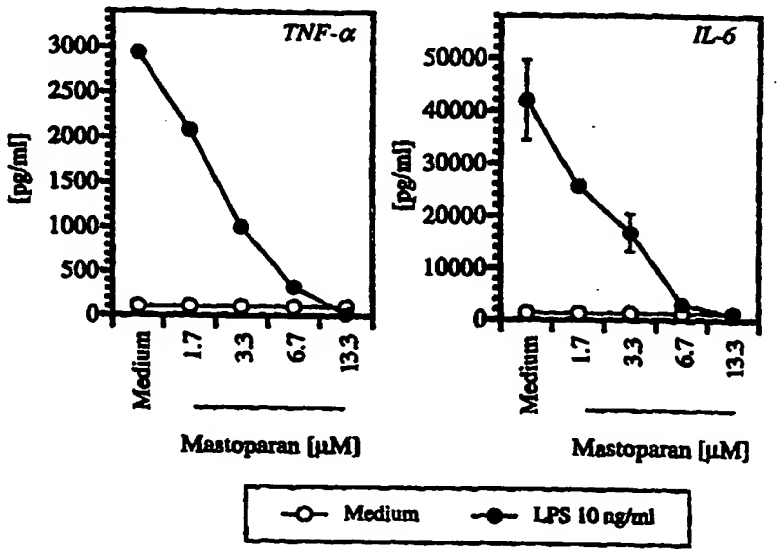
(54) Title: THE USE OF AGENTS WHICH BIND G PROTEINS FOR TREATING SEPTIC SHOCK



(57) Abstract

The present invention provides for the use of G protein binding agents for prophylactic and/or therapeutic treatment of septic shock. The present invention provides methods of using agents which bind G protein to treat a subject having or susceptible to septic shock. The present invention further pertains to compositions for treating a subject for septic shock. The composition includes an effective amount of a G protein binding agent such as mastoparan and, optionally, an antibiotic and a pharmaceutically acceptable carrier. Other aspects of the invention include packaged agents which bind G proteins for treating septic shock.

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COMPOSITIONS AND METHODS FOR CONTRACEPTION IN OR STERILIZATION OF MAMMALS

The benefit of the September 3, 1997 filing date of provisional application 60/057,456 is claimed under 35 U.S.C. § 119(e) in the United States, and is claimed under applicable treaties and conventions outside the United States.

TECHNICAL FIELD

This invention pertains to compositions and methods for long-term contraception or sterilization of mammals.

BACKGROUND ART

Compositions that have sometimes been used for long-term contraception include those based upon natural or synthetic steroidal hormones to "trick" the female reproductive tract into a "false pregnancy." These steroidal hormones must be administered repeatedly to prevent completion of the estrous cycle and conception. Steroids have side effects that can be potentially dangerous.

P. Olson *et al.*, "Endocrine Regulation of the Corpus Luteum of the Bitch as a Potential Target for Altering Fertility," *J. Reprod. Fert. Suppl.*, vol. 39, pp. 27-40 (1989) discusses the luteal phase and its regulation in bitches. The following discussion appears at page 37: "Specific toxins can be linked to an antibody or hormone and carried to a specific target cell (or cells) which is then killed by the toxin. The idea of developing a 'magic bullet' has been discussed for decades but is now gaining renewed recognition as a potential, highly selective method for destroying specific tissues while leaving other tissues unharmed. For many years it was impossible to develop large quantities of antibodies which would react specifically with only single antigenic determinants. However, with the advent of monoclonal antibodies, this problem has been largely overcome. Antibodies can be developed to specific

hormone receptors (such as the LH receptor) and then coupled to a toxin. All cells with LH receptors should then be destroyed. Although various cell types have not been characterized in dog corpora lutea, destruction of any luteal cell type could potentially result in luteolysis if cell types communicate." (citations omitted)

P. Olson *et al.*, "New Developments in Small Animal Population Control," *JAVMA*, vol. 202, pp. 904-909 (1993) gives an overview of methods for preventing or terminating unwanted pregnancies in small animals. The following discussion appears at page 905: "*Tissue-specific cytotoxins*--Permanent contraception in females and males might be achieved by administration of a cytotoxin that is linked to gonadotropin-releasing hormone (GnRH) and that selectively destroys gonadotropin-secreting pituitary cells. Similarly, a cytotoxin linked to antibodies against gonadotropin receptors could be targeted to alter gonadal function. Toxins would need to be carefully targeted to specific cells, yet be safe for all other body tissues." (citation omitted).

T. Janaky *et al.*, "Short Chain Analogs of Luteinizing Hormone-Releasing Hormone Containing Cytotoxic Moieties," *Proc. Natl. Acad. Sci. USA*, vol. 89, pp. 10203-10207 (1992) discloses the use of certain hexapeptide and heptapeptide analogs of GnRH as carriers for certain alkylating nitrogen mustards, certain anthraquinone derivatives, antimetabolite, and cisplatin-like platinum complex.

S. Sealfon *et al.*, "Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor," *Endocrine Reviews*, vol. 18, pp. 180-205 (1997) provides a review of research concerning the interaction between GnRH and its receptor.

D. Morbeck *et al.*, "A Receptor Binding Site Identified in the Region 81-95 of the β-Subunit of Human Luteinizing Hormone (LH) and chorionic gonadotropin (hCG)," *Molecular and Cellular Endocrinology*, vol. 97, pp. 173-181 (1993) disclosed a fifteen amino acid region of LH and hCG that acted as a receptor binding site. (LH and hCG are homologous hormones that produce similar effects.)

S. Cho *et al.*, "Evidence for autocrine inhibition of gonadotropin-releasing hormone (GnRH) gene transcription by GnRH in hypothalamic GT1-1 neuronal cells," *Mol. Brain Res.*, vol. 50, pp. 51-58 (1997) discloses that neuroendocrine populations of GnRH neurons have high affinity receptors for GnRH and for GnRH analogs.

N. Mores *et al.*, "Activation of LH receptors expressed in GnRH neurons stimulates cyclic AMP production and inhibits pulsatile neuropeptide release," *Endocrinology*, vol. 137, pp. 5731-5734 (1996) discloses that LH acts directly on neuroendocrine neurons in the brain. See also Z. Lei *et al.*, "Signaling and transacting factors in the transcriptional inhibition of

gonadotropin releasing hormone gene by human chorionic gonadotropin in immortalized hypothalamic GT1-7 neurons," *Mol. & Cell. Endocrinology*, vol. 109, pp. 151-157 (1995).

Conventional targeted toxin therapies have several drawbacks. There is a small window for treatment with a particular targeted toxin (on the order of two weeks) before the recipient's immune system mounts an antibody response to the targeted toxin. These antibodies will neutralize the toxin; or worse, may result in the deposition of the toxin in reticuloendothelial tissues (e.g., liver, spleen, lymph nodes, lungs, bone marrow), where they may damage otherwise healthy tissue. Aside from this drawback, the toxin must be internalized by the targeted cell and translocated into the cytoplasm to have effect.

U.S. Patents No. 5,378,688; 5,488,036; and 5,492,893 disclose compounds said to be useful in inducing sterility in mammals. The disclosed compounds were generically described as GnRH (or a GnRH analog) conjugated to a toxin. The toxin was preferably linked to the sixth amino acid of the GnRH agonist. The toxin was preferably one with a translocation domain to facilitate uptake into a cell. The inventors noted that conjugation of the GnRH agonist to the toxin "is necessary because, for the most part, the above toxins, by themselves, are not capable of binding with cell membranes in general. That is to say that applicants have found that it is only when a GnRH analog of the type described herein is linked to a toxin of the type noted above does that toxin become capable of binding to cell membranes . . . ." (E.g., Pat. No. 5,488,036, col. 7, lines 46-52.) The toxins specifically mentioned appear all to have been metabolic toxins, for example ricin, abrin, modeccin, various plant-derived ribosome-inhibiting proteins, pokeweed antiviral protein, α-amanitin, diphtheria toxin, pseudomonas exotoxin, shiga toxin, melphalan, methotrexate, nitrogen mustard, doxorubicin, and daunomycin. None of these toxins is believed to be toxic due to direct interaction with the cell membrane. In the *in vivo* experiments reported, the most effective time course was reported to be weekly injections for 4 weeks. (E.g., Pat. 5,488,036, col. 20, lines 46-47.) Because most of the conjugates cited are relatively large compounds, antigenicity could be a problem when such multiple administrations are used. The GnRH analog was preferably linked to the toxin with one of several specified heterobifunctional reagents. The specifications suggest that considerable effort was expended in conjugating the toxin to the GnRH agonist. The toxins must in general be internalized into the target cells to have effect, and do not act on cell membranes; in addition, at least some of these toxins must be secondarily transported from the membrane-bound vesicle into the cytoplasm to interact with ribosomes, mitochondria, or other cellular components.

DISCLOSURE OF INVENTION

It has been unexpectedly discovered that amphipathic lytic peptides are ideally suited to use in a ligand/cytotoxin combination to specifically induce sterility or long-term contraception in mammals. The peptides act directly on cell membranes, and need not be internalized. Administering a combination of gonadotropin-releasing hormone (GnRH) (or a GnRH agonist) and a membrane-active lytic peptide produces long-term contraception or sterilization in mammals *in vivo*. Particularly surprising, sterility results even when the combination is administered to a sexually immature animal: The combination then prevents sexual maturation.

The compounds used in the present invention are relatively small, and will not be antigenic. (Lytic peptides are known not to be very antigenic; and the ligands are not antigenic at all.) The compounds may be administered in a single dose, although they may also be given in two or more closely spaced doses. Lysis of gonadotropes has been observed to be very rapid (on the order of ten minutes.) The two components -- the ligand and the lytic peptide -- may optionally be administered as a fusion peptide, or they may be administered separately, with the ligand administered slightly before the lytic peptide, to activate cells with receptors for the ligand, and thereby make those cells susceptible to lysis by the lytic peptide. If a fusion peptide is used, it has been unexpectedly discovered that a linking moiety is not necessary to join the ligand to the lytic peptide: one may be bonded directly to the other, without the need for any intervening linkage; bonding is preferably performed by bonding one end of the ligand to one end of the peptide, not by bonding to the middle of either. The toxin, the lytic peptide, does not need a translocation domain, and need not be internalized, as it binds to and acts directly on the activated cell membrane to cause lysis.

MODES FOR CARRYING OUT THE INVENTION

It is known that the D-amino acid form of GnRH will bind to gonadotropes in the pituitary and to GnRH neurons in the brain. It is also known that the D-amino acid forms of lytic peptides have essentially the same propensity to lyse cell membranes as do the L-amino acid forms. Compounds of the present invention (whether administered as a fusion peptide or separately) may therefore be administered either in L-form or D-form. D-form peptides, although generally more expensive than L-form, have the advantage that they are not degraded by normal enzymatic processes, so that the D-form peptides may therefore be administered orally and generally have a longer biological half-life. Oral administration of the D-form peptide may be enhanced by linking the peptide/hormone fusion product to a suitable carrier to facilitate uptake by the intestine, for example vitamin B_{12}, following generally the

B_{12}-conjugation technique of G. Russell-Jones *et al.*, "Synthesis of LHRH Antagonists Suitable for Oral Administration via the Vitamin B_{12} Uptake System," *Bioconjugate Chem.*, vol. 6, pp. 34-42 (1995).

GnRH or GnRH analogs (collectively, "GnRH agonists") may be used in the present invention. It has been reported that substitutions at the 6 and 10 positions of the GnRH decapeptide can produce "superagonists" having greater binding affinity to the GnRH receptor than does GnRH itself. These "superagonists" include goserelin, leuprolide, buserelin, and nafarelin. See U.S. Patent 5,488,036.

Without wishing to be bound by this theory, it is believed that the mechanism underlying the invention is as follows: GnRH activates gonadotropic cells in the pituitary gland, as well as neuroendocrine GnRH neurons in the brain. The activated cells have substantially increased susceptibility to lysis by a lytic peptide. The lytic peptide then preferentially destroys (or severely damages) these activated cells. When the gonadotrophic cells in the pituitary are destroyed and are deprived of GnRH from the brain, the pituitary no longer secretes follicle stimulating hormone (FSH) or luteinizing hormone (LH), rendering the mammal temporarily or permanently sterile.

Although the ligand and the lytic peptide may be administered separately, it is preferred to link the two in a single molecule, because such a linkage greatly increases the effective concentration of the lytic peptide in the vicinity of ligand-activated cells. Furthermore, this increase in the effective lytic peptide concentration can obviate the need for activation of the cells, allowing the peptide to be linked to a binding site of a ligand alone, without needing to include the "remainder" of a native ligand that would normally be needed for activating the target cells. This linkage may be in either order: for example, GnRH/peptide or peptide/GnRH. Examples are GnRH/hecate (SEQ. ID NO. 3) and hecate/GnRH (SEQ. ID NO. 4). Note that no intermediate linker is necessary, and that the carboxy terminus of one of the two peptides may be bonded directly to the amino terminus of the other. (We have found that the initial pyro-glutamic acid residue of the GnRH or the GnRH portion of a fusion peptide may be substituted with glutamine without substantially changing the activity of the respective peptides. See, e.g., SEQ. ID Nos. 9, 3, and 4.)

Experimental Results

Examples 1-6

The pituitary gland of an adult female rat was harvested and divided into six sections of approximately equal size. One section was placed in each of six wells containing tissue culture medium at 37°C. A different treatment was applied to each well, as described below. Ten hours after treatment, the tissue from each well was fixed, and the histology of each was examined microscopically.

Treatment 1 applied tissue culture medium alone as a control. The histology of this tissue after treatment appeared normal.

Treatment 2 was an application of 5 nanograms of GnRH (SEQ. ID NO. 1) per mL of medium. The histology of this tissue after treatment was normal; a small degree of cellular vacuolization was noted. For comparison, the concentration of GnRH in normal, untreated rats varies from as low as 1 ng/mL to as high as 20 ng/mL during the LH surge phase of the estrous cycle.

Treatment 3 was an application of 50 μM of the lytic peptide hecate (SEQ. ID NO. 2) in the medium. The histology of this tissue after treatment appeared normal.

Treatment 4 was an initial application of 5 nanograms of GnRH per mL of medium for 15 minutes. Following this incubation, the medium containing GnRH was removed, and the tissue was washed once with plain medium. This medium was then removed, and was replaced with medium containing 50 μM of the lytic peptide hecate. Widespread basophilic (gonadotropic) cellular destruction was observed after this treatment.

Treatment 5 was an application of 50 μM of the fusion peptide modified GnRH/hecate (SEQ. ID NO. 3). Widespread basophilic (gonadotropic) cellular destruction was observed after the treatment.

Treatment 6 was an initial application of the fusion peptide GnRH/hecate (SEQ. ID NO. 3), followed by a second application of the fusion peptide GnRH/hecate two hours later. After treatment the tissue was virtually destroyed, with only stromal cells remaining.

Example 7

Two sexually immature female rats from the same litter (age 33 days) were given two intravenous injections of saline control solution 24 hours apart. After the rats reached breeding age, they were examined 105 days post-inoculation. The external genitalia appeared normal. During a fourteen-day monitoring period 107 days to 121 days post-inoculation, each of the control rats completed two estrous cycles. The rats were then sacrificed and necropsied. The reproductive organs appeared histologically normal.

Example 8

Two sexually immature female rats from the same litter as those of Example 7 (age 33 days) were given two intravenous injections of 500 μg GnRH/hecate fusion peptide in saline 24 hours apart. After the rats reached breeding age, they were examined 105 days post-inoculation. The external genitalia appeared small. Unlike the control rats, insertion of a cotton-tipped swab into the vagina was difficult. During a fourteen-day monitoring period 107 days to 121 days post-inoculation, neither of the treated rats demonstrated estrous or metestrous. The rats were then sacrificed and necropsied. The peptide-treated rats had thinned, inactive uterine and oviductal epithelia. Their ovaries contained no large follicles, and had a high number of atretic follicles (i.e., those that had failed to ovulate).

Examples 9-14

Eighteen sexually mature, mixed breed, female rats were randomly assigned to one of six groups containing three rats each. Each group of rats received a double treatment intravenously, as described below. Two weeks after the treatment, the rats were sacrificed and necropsied. The reproductive and endocrine organs were sectioned, weighed, and examined histologically.

Treatment 9 was a saline control. The rats in this group exhibited normal ovarian function (e.g., normal follicles and new corpora lutea). The pituitaries from this group were of normal size. Histology showed a normal number of pituitary basophilic cells.

Treatment 10 was injection with a total of 1.0 mg GnRH/hecate fusion peptide in saline, divided into two equal 0.5 mg injections administered 24 hours apart. The rats in this group showed an arrest of normal ovarian follicular development. Few corpora lutea were present, and those that were present appeared old. Follicles were large, and had not ruptured. Uterine morphology was consistent with hormonal inactivity. The pituitaries from this group were slightly smaller than the pituitaries from the saline control group. Histology revealed a 60% to 70% reduction in the number of pituitary basophilic cells compared to the controls.

Treatment 11 was injection of 100 μL of a 1.35 mM solution of GnRH (162 μg) in saline, followed 15 minutes later by injection with 100 μL of a 1.35 mM solution of hecate (337 μg) in saline. The same two-step treatment was repeated 24 hours later. The rats in this group showed altered ovarian histology. Few corpora lutea were present, and those that were present appeared old. Follicles were large, and had not ruptured. Uterine morphology was consistent with hormonal inactivity. The pituitaries and the pituitary histology were similar to those observed in Treatment 10.

Treatment 12 was injection of 100 μL of a 1.35 mM solution of hecate (337 μg) in saline. The treatment was repeated after 24 hours. The rats in this group exhibited normal ovarian function (e.g., normal follicles and new corpora lutea). The pituitaries and the pituitary histology were similar to those observed in Treatment 9.

Treatment 13 was injection of 100 μL of a 1.35 mM solution of GnRH (162 μg) in saline. The treatment was repeated after 24 hours. The rats in this group exhibited normal ovarian function (e.g., normal follicles and new corpora lutea). The pituitaries and the pituitary histology were similar to those observed in Treatment 9.

Treatment 14 was identical to Treatment 10, except that the GnRH/hecate fusion peptide was further purified by HPLC. The rats in this group showed an arrest of normal ovarian follicular development. Few corpora lutea were present, and those that were present appeared old. Follicles were large, and had not ruptured. Uterine morphology was consistent with hormonal inactivity. The pituitaries and the pituitary histology were similar to those observed in Treatment 10.

These experiments demonstrate that GnRH and the lytic peptide may be administered either separately or as a fusion peptide, although the fusion peptide is preferred as it is expected to be more active at lower doses.

Although experiments to determine optimum dosages had not been performed by the time this application is being filed, a person of ordinary skill in the art, who is given the teachings of the present specification, may readily ascertain optimum dosages through routine testing.

Although the experiments to date have been performed on female mammals, similar results are expected for male mammals, because GnRH signals pituitary cells to release gonadotropins in both males and females.

Tissue and cell specificity of cytotoxic conjugates could be further enhanced by using various hormones or hormone analogs coupled to a lytic peptide. Some examples follow. For fertility control, both the pituitary and the central GnRH neuronal component of the reproductive axis are selectively damaged by GnRH-hecate conjugate. Few cells in the central nervous system should be damaged by this treatment, because the chicken II GnRH and lamprey III GnRH forms are the primary molecules affecting brain function in most mammals. Fertility control may also be selectively accomplished by treating animals with a bLH-hecate

conjugate; this compound should specifically affect GnRH neurons controlling reproduction and the gonads. (Other lytic peptides may be used in place of hecate in these conjugates.)

The compositions of the present invention may be administered as described, or as pharmaceutically acceptable salts. The compositions may be administered intravenously, subcutaneously, intramuscularly, or (especially when in D-amino acid form and complexed with a carrier such as vitamin B_{12}) orally.

Applications of the present invention include long-term contraception or sterilization in humans; and long-term contraception or sterilization in domesticated or wild mammals. Domesticated mammals in which this invention may be used include, for example, dogs, cats, cattle, horses, pigs, and sheep. When used in humans, long-term replacement hormone therapy may be needed to prevent undesirable side effects, such as premature menopause. Administration of gonadotropic hormones in a sterilized individual will temporarily restore fertility if desired. The sterilization is reversible in this sense.

As one example, this invention may be used in the humane population control of an unwanted introduced species.

Examples 15-22

Eight sexually mature, Sprague-Dawley female rats were randomly assigned to one of eight treatments. Each group of rats received a single treatment intravenously, as described below. Rats were sacrificed and necropsied either 48 or 96 hours after treatment. The ovaries, uterus, pancreas, liver, spleen, lungs, heart, thyroid, and adrenal glands were fixed in 10% buffered formalin; sectioned; and stained with H&E (hematoxylin and eosin) stain; except that the pituitary glands were stained with PAS (periodic acid-Schiff) stain with no counter-stain. The treatments were selected so that each animal received an equimolar amount of the compound with which it was treated.

Treatments 15 and 16 were IV-injection with 674 μg of D-hecate in 200 μL saline (1.35 mM). The rat in treatment 15 was sacrificed 48 hours after injection, and the rat in treatment 16 was sacrificed 96 hours after injection. No gross lesions were noted in the organs of either animal. The pituitary glands of both rats contained a normal number of PAS-positive cells.

Treatments 17 and 18 were IV-injection with 334 μg of GnRH in 200 μL saline (1.35 mM). The rat in treatment 17 was sacrificed 48 hours after injection, and the rat in treatment 18 was sacrificed 96 hours after injection. No gross lesions were noted in the organs of either animal. The pituitary glands of both rats contained a normal number of PAS-positive cells.

Treatments 19-22 were IV-injection with 1 mg GnRH-hecate fusion peptide (SEQ. ID NO. 3) in 100 μL saline (2.7 mM). The rats in treatments 19 and 20 were sacrificed 48 hours after injection, and the rats in treatments 21 and 22 were sacrificed 96 hours after injection. No gross lesions were noted in the organs of any of the four animals, other than the pituitary. The pituitary glands of the animals from treatments 19 and 20 were slightly enlarged, hyperemic, and edematous. The pituitary glands of the animals from treatments 21 and 22 were slightly hyperemic, but of expected size. The pituitary glands of all four rats showed a marked depletion of PAS-positive cells; it was estimated that the depletion was 80 to 90% compared to those of control groups. (PAS stain preferentially stains glycopeptides. LH, FSH, and MSH are glycopeptide hormones; cells containing these hormones stored in their secretory vacuoles stain positive with PAS.)

It was thus seen that the GnRH-lytic peptide combination caused morphological and functional alterations in the adult female rat reproductive system, and in preventing sexual maturity in pre-pubertal female rats, but that the fusion peptide selectively eliminated a specific population of PAS-positive staining cells in the pituitary.

Example 23

Subsequent experiments were conducted on rats using treatments generally similar to treatments 9-14 above. Observations made with immunohistochemical staining found that the effective treatments selectively killed **(1)** gonadotropes in the pituitary, and **(2)** neurons in the brain bearing GnRH receptors. The selective killing of these cells was seen after the GnRH-hecate fusion peptide was administered; and after the administration of GnRH alone, followed 10 minutes later by the administration of hecate alone. In these cases, it was also observed that pituitary cells no longer secreted either LH or FSH following the effective treatments.

Example 24

Hecate is an amphipathic lytic peptide that acts on cell membranes without being internalized. It is a synthetic peptide analog of melittin, the primary toxin in honeybee

venom. Hecate is believed to act by disrupting cell membranes. The structure of the modified GnRH-hecate conjugate used in these studies was SEQ. ID NO. 3.

We also synthesized D-Lys^{6}GnRH (SEQ. ID NO. 13), so that hecate could be conjugated to the D-Lys^6, a position that could minimize interference with binding of the GnRH domain to the GnRH receptor. These synthetic peptides specifically displaced radiolabelled monoiodinated-GnRH from rat pituitary membranes. Displacement by D-Lys^{6}GnRH-hecate was comparable to (and actually slightly greater than) displacement by native mammalian GnRH, as measured by cpm of radioactivity. When GnRH and GnRH-hecate binding were compared on a molar basis over a 1000-fold concentration range (n = 6) the GnRH-hecate specifically displaced the radiolabelled peptide to an extent equal to 123% ± 4% of the binding exhibited by equimolar concentrations of GnRH; equimolar concentrations of D-Lys^{6}GnRH displaced 187% ± 8% of the cpm displaced by native GnRH.

Examples 25-32

We studied *in vitro* lysis of bovine luteal cells with GnRH-hecate conjugate and with hecate-bLH conjugate (SEQ. ID NO. 12). (The bLH component of the conjugate is a 15-mer fragment of the beta chain of luteinizing hormone, SEQ. ID NO. 11). Small luteal cells were collected from cattle corpora lutea post-slaughter. Small luteal cells are rich in LH receptors, and were found to be highly susceptible to lysis by the hecate-bLH conjugate.

Small luteal cells in culture were incubated with one of the following treatments for 22 hours, and were then examined for viability using Trypan Blue exclusion and release of lactic dehydrogenase.

Treatment 25 control: no additional treatment (media alone)

Treatment 26 10 ng bLH (positive control)

Treatment 27 hecate-bLH, 10 μM

Treatment 28 hecate-bLH, 5 μM

Treatment 29 hecate-bLH, 1 μM

Treatment 30 hecate (alone), 10 μM

Treatment 31 hecate (alone), 5 μM

Treatment 32 hecate (alone), 1 μM

Significant killing of small luteal cells was observed following 22 hr. incubation with 10 μM hecate alone, and with 5 μM hecate alone (approximately 50% killing). Cell death for 1 μM hecate alone did not differ significantly from negative control (media) or from bLH alone. All three treatment doses with hecate-bLH caused significant increases in cell death as

compared to treatment with hecate alone. The hecate-bLH conjugate killed approximately twice the number of cells as were killed by hecate alone at the same concentrations.

Observed levels of lactic dehydrogenase activity also demonstrated that the hecate-bLH treatment killed a significantly greater number of cells than did hecate alone.

Examples 33-34

We also studied *in vitro* lysis of bovine granulosa cells with GnRH-hecate conjugate and with hecate-bLH conjugate. Granulosa cells were isolated from bovine pre-ovulatory follicles. (Granulosa cells are hormonally active cells with numerous LH receptors.) Our experiments with granulosa cells were otherwise generally similar to those described above for Examples 25-32. These experiments demonstrated (1) that the granulosa cells were much more susceptible to killing by hecate alone than were the small luteal cells, and (2) that, as had been the case with the small luteal cells, the granulosa cells were significantly more susceptible to hecate-bLH at even the lowest concentration (1 μM) than they were to hecate alone. At 1 μM, the hecate-bLH conjugate killed about twice the number of target cells as did hecate alone. Again, the levels of lactic dehydrogenase released following the hecate-bLH 1 μM treatment were significantly higher than the levels of enzyme released following treatment with 1 μM hecate alone.

Additional studies (data not shown) demonstrated that a 15-mer fragment of the bLH subunit specifically bound to LH receptors on the target granulosa cells, but did not initiate the production of steroid hormones that would be indicative of a stimulus-coupled response. We thus demonstrated that the selective killing of target cells resulted from the physical proximity of the lytic peptide to the cell, which was caused by binding of the LH subunit. Stimulation of target cell hormone production was not required for cell lysis. This result was surprising, as we had previously expected that activation of the target cells was required for increased susceptibility to lysis. These data demonstrate that such activation is not required. These data are, however, consistent with our other data showing that cell activation is also a route that can lead to increased susceptibility to the lytic peptide.

Examples 35-38

Another set of experiments was performed to study the *in vivo* effects of the GnRH-hecate conjugate on female rats and rabbits. The ovaries, uterus, oviducts, adrenals, spleen, thyroids, pancreas, liver, lungs, and heart were processed for histological analysis. The pituitaries were processed for histological analysis of PAS-stained cells and for cells stained immunocytochemically for bLH, BFSH (bovine follicle stimulating hormone),

adrenocorticotropic hormone, and other proopiomelanocortin peptide products (most notably alpha-melanocyte stimulating hormone (MSH)), thyroid stimulating hormone (TSH), prolactin (PRL), vasopressin (VP), oxytocin (OXY) or growth hormone (GH). The immunocytochemical staining procedures we used followed generally the procedures of M. Rahmanian *et al.*, "Histological and immunocytochemical characterization of pituitary cell types in ponies," *Proc. 13th Soc. Equine Nutrition & Phys. Symp.*, pp. 348-349 (1993); M. Rahmanian *et al.*, "Immunocytochemical localization of luteinizing hormone and follicle-stimulating hormone in the equine pituitary," *J. Anim. Sci.*, vol. 76, pp. 839-846 (1998); M. Rahmanian *et al.*, "Immunocytochemical localization of prolactin and growth hormone in the equine pituitary," *Animal Sci.*, vol. 75, pp. 3010-3018 (1997); and P. Melrose *et al.*, "Comparative topography of the immunoreactive alpha-melanocyte-stimulating hormone neuronal system in the brains of horses and rats," *Brain Beh. & Evol.*, vol. 32, pp. 226-235 (1988).

Brains were serially sectioned on a Vibrotome from the level of the diagonal band of Broca to the mammillary body. Alternate sections were consecutively divided into four to five dishes, and sections in alternate dishes were stained with cresyl violet, or were stained immunocytochemically for GnRH or the GnRH precursor, VP, OXY, or tyrosine hydroxylase (the rate-limiting enzyme in catecholamine synthesis). In addition to the staining procedures cited above, we also used the immunocytochemical staining procedures of P. Melrose *et al.*, "Distribution and morphology of immunoreactive gonadotropin-releasing hormone (GnRH) neurons in the basal forebrain of ponies," *J. Comp. Neurol.* vol. 339, pp. 269-287 (1994); and P. Melrose *et al.*, "Topography of oxytocin and vasopressin neurons in the forebrain of Equus caballus: Further support of proposed evolutionary relationships for proopiomelanocortin, oxytocin and vasopressin neurons," *Brain, Beh. & Evol.*, vol. 33, pp. 193-204 (1989).

Thirty-three-day-old, sexually immature female rats were given intravenous administrations as follow:

Treatment 35: 0.03 μg GnRH (a normal physiological dose) (two rats)

Treatment 36: 1.62 μg GnRH (the molar equivalent to the amount of GnRH in Treatment 37) (one rat)

Treatment 37: 0.5 mg GnRH-hecate (one rat)

Treatment 38: 0.03 μg GnRH, followed 11 minutes later by 0.337 μg hecate (two rats).

Animals were sacrificed 14 days after treatment. As compared to the two GnRH control groups, the treatment with GnRH-hecate and the treatment with GnRH followed by

hecate alone reduced pituitary weights by 13% and 14%, respectively, and reduced the numbers of bLH-specific gonadotropes by 92% and 87%, respectively. Further, following these two experimental treatments the cell bodies of GnRH-stained neurons in hypophysiotropic areas of the brain were frequently deformed; and a substantial amount of immunoreactive material leached into surrounding areas where numerous cell bodies are concentrated (the organum vasculosum of the lamina terminalis). There was histological damage to cells from the two experimental treatments in the C1 and C3 fields of the hippocampus, and increased staining of parvicellular VP neurons in the paraventricular nucleus. (The VP staining may have been caused by formation of a precipitate in certain areas of the brain. Subsequent studies with more highly purified peptide did not show a precipitate). The change in VP expression, probably in corticotropin-releasing neurons, may cause a shift in the post-translational processing of proopiomelanocortin peptide products in the pars distalis, since GnRH-hecate and GnRH + hecate treatments reduced adrenocorticotropic hormone levels and increased the number of alpha-MSH-stained cells in this subdivision of the pituitary. No pathological changes were noted in any other tissues.

Since neurons in the brain do not regenerate, severe damage to these neurons could make sterilization with a GnRH/lytic peptide combination permanent (but temporarily reversible by administration of gonadotrophic hormones).

Examples 39-43

Sexually immature (33 day old) female rats (randomly allocated into groups of three) were injected intravenously with saline or GnRH-hecate in saline as follows:

Treatment 39: 0.0 mg GnRH-hecate

Treatment 40: 0.1 mg GnRH-hecate

Treatment 41: 0.5 mg GnRH-hecate

Treatment 42: 1.0 mg GnRH-hecate

Treatment 43: 1.5 mg GnRH-hecate.

Animals were sacrificed at 24 hours or at 14 days after treatment. Results were similar to those reported above for Examples 35-38, except that no precipitate was found in the brain, and VP staining in the CNS was not altered. The treatments with higher levels of GnRH-hecate produced a large number of GnRH-receptor-containing neurons having abnormal morphologies, including distortion of the somatic portion of the cells, and degeneration of neurites. In the rats sacrificed fourteen days after treatment, 66% and 87% of the GnRH-receptor-containing neurons were abnormal in the rats that had received 1.0 and 1.5 mg of

GnRH-hecate, respectively. Axonal degeneration in the 1.5 mg GnRH-hecate group was accompanied by over 90% reduction in median eminence staining for GnRH.

Examples 44-46

Seven sexually mature female New Zealand rabbits were injected intravenously with saline containing GnRH-hecate as follows:

Treatment 44: 0 mg GnRH-hecate (n = 3)

Treatment 45: 5 mg GnRH-hecate (n = 3)

Treatment 46: 10 mg GnRH-hecate (n = 1).

Forty-six days later all rabbits were injected intramuscularly with 100 μg GnRH. Blood samples were collected at 0, 1, 4, and 24 hours, and LH and FSH levels in the blood samples were measured by radioimmunoassay. Hormone analyses revealed that both control and experimental animals released LH in response to the GnRH, suggesting that there may be at least some degree of reversibility following treatment, at least for pituitary gonadotropes at lower doses of ligand/peptide. The rabbits were sacrificed the next day (day 47) for postmortem histological analysis. We found that the numbers of tertiary follicles, corpora lutea, and GnRH-induced ovulations were reduced by GnRH-hecate treatment. Ovarian and pituitary weights were reduced by the 10 mg GnRH-hecate treatment. In tissues from the GnRH-hecate treatments, observed immunoreactive GnRH was faint and diffusely localized in CNS areas normally containing cell bodies; normal individual cell bodies were reduced in number by at least 50%; and the terminal fields, which normally contain the axons of GnRH receptor neurons, were not stained for GnRH. These observations suggest that the most pronounced effects of the GnRH-hecate treatments in these experiments on rabbits may have been on neuroendocrine neurons in the brain. The hippocampus and other areas of the brain containing high concentrations of GnRH were not discernibly affected by GnRH-hecate treatments. The GnRH-hecate treatment increased the number of PAS-stained pituitary cells in the pars distalis to 177% of that for control rabbits; this increase appeared to reflect increased numbers of cells staining alpha-MSH, and reduced numbers of cells staining for LH.

Examples 47-48

Nine sexually mature female rabbits were injected intravenously with saline containing 0 mg (n = 4) (Treatment 47) or 10 mg GnRH-hecate (n = 5) (Treatment 48). Rabbits were injected intramuscularly with GnRH on day 6 posttreatment. Blood samples were collected for radioimmunoassay of LH and FSH as described above, and the animals were sacrificed on day 7 post-treatment. Both control and experimental animals released LH in response to the

GnRH; however, the amount of LH released was lower in the treated animals than in the controls. The GnRH-hecate treatment reduced the numbers of tertiary ovarian follicles, and the numbers of GnRH-induced ovulations. No effects were noticed either on peripheral tissues or on pituitary weight. The effects of GnRH-hecate on CNS morphology and immunocytochemical results were similar to those described above in Examples 35-46. Again, the effects were more pronounced on GnRH neurons than on staining of pituitary gonadotropes.

The number of ovulation sites in rabbits in Examples 47 and 48 treated with 10 mg GnRH-hecate were reduced as compared to saline controls. The mean number of ovulation sites in four saline controls equalled 12.2 $\pm$ 5.4, with S.E.M. = 2.7. The mean number of ovulation sites in the five rabbits given 10 mg of GnRH-hecate was 3.6 $\pm$ 1.1, with S.E.M. = 0.5. This difference from control was significant ($p = 0.025$).

The "LH surge" (the level of LH at one hour post-GnRH challenge, minus the resting level before challenge) in the four controls was 61.2 $\pm$ 16.5 ng/mL, with S.E.M. = 8.3; and in the treated group was 49.6 $\pm$ 26.1 ng/mL, with S.E.M. = 12 ($p = 0.22$). Thus there was a trend towards lower LH levels in the treated group.

The *in vivo* studies clearly demonstrated that the GnRH-hecate conjugate selectively damaged GnRH receptor-bearing cells in the brain (neurons) and in the pituitary (gonadotrophic cells). Further, these studies demonstrated a significant alteration in the ovary, presumably a consequence of alteration in the reproductive centers of the brain-pituitary axis. Selectivity of the conjugate was demonstrated by the following observations: (1) No cytotoxic changes were seen in neurons that lacked GnRH receptors. (2) No changes were seen in pituitary cells that lacked GnRH receptors. (3) No changes were seen in other endocrine and non-endocrine tissues (except for the ovary, which presumably responded indirectly to the destruction of gonadotrophs in the pituitary).

Many of the events referred to as "ovulations" in the GnRH-hecate treated rabbits possibly were not functional ovulation sites, but may instead have represented hemorrhagic pre-ovulatory degenerative changes. Additional breeding trials will be conducted to verify that ovulation of functional ova is prevented.

Lytic Peptides Useful in the Present Invention

It is believed (without wishing to be bound by this theory) that lytic peptides act by disrupting cell membranes. "Resting" eukaryotic cells protect themselves through their ability to repair the resulting membrane damage. By contrast, activated cells (e.g., cells stimulated

by GnRH) are unable (or less able) to repair damaged membranes. Because GnRH-activated pituitary cells have a diminished capacity to repair membranes, they are preferentially destroyed by lytic peptides, while adjacent non-activated cells repair their membranes and survive.

Although the embodiments of this invention that have been tested to date have used hecate as the effector lytic peptide, this invention will work with a combination of GnRH with other lytic peptides as well. Many lytic peptides are known in the art and include, for example, those mentioned in the references cited in the following discussion.

Lytic peptides are small, basic peptides. Native lytic peptides appear to be major components of the antimicrobial defense systems of a number of animal species, including those of insects, amphibians, and mammals. They typically comprise 23-39 amino acids, although they can be smaller. They have the potential for forming amphipathic alpha-helices. See Boman *et al.*, "Humoral immunity in *Cecropia* pupae," *Curr. Top. Microbiol. Immunol.* vol. 94/95, pp. 75-91 (1981); Boman *et al.*, "Cell-free immunity in insects," *Annu. Rev. Microbiol.*, vol. 41, pp. 103-126 (1987); Zasloff, "Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial DNA sequence of a precursor," *Proc. Natl. Acad. Sci. USA*, vol. 84, pp. 3628-3632 (1987); Ganz *et al.*, "Defensins natural peptide antibiotics of human neutrophils," *J. Clin. Invest.*, vol. 76, pp. 1427-1435 (1985); and Lee *et al.*, "Antibacterial peptides from pig intestine: isolation of a mammalian cecropin," *Proc. Natl. Acad. Sci. USA*, vol. 86, pp. 9159-9162 (1989).

Known amino acid sequences for lytic peptides may be modified to create new peptides that would also be expected to have lytic activity by substitutions of amino acid residues that preserve the amphipathic nature of the peptides (e.g., replacing a polar residue with another polar residue, or a non-polar residue with another non-polar residue, etc.); by substitutions that preserve the charge distribution (e.g., replacing an acidic residue with another acidic residue, or a basic residue with another basic residue, etc.); or by lengthening or shortening the amino acid sequence while preserving its amphipathic character or its charge distribution. Lytic peptides and their sequences are disclosed in Yamada *et al.*, "Production of recombinant sarcotoxin IA in *Bombyx mori* cells," *Biochem. J.*, vol. 272, pp. 633-666 (1990); Taniai *et al.*, "Isolation and nucleotide sequence of cecropin B cDNA clones from the silkworm, *Bombyx mori*," *Biochimica Et Biophysica Acta*, vol. 1132, pp. 203-206 (1992); Boman *et al.*, "Antibacterial and antimalarial properties of peptides that are cecropin-melittin hybrids," *Febs Letters*, vol. 259, pp. 103-106 (1989); Tessier *et al.*, "Enhanced secretion

from insect cells of a foreign protein fused to the honeybee melittin signal peptide," *Gene*, vol. 98, pp. 177-183 (1991); Blondelle *et al.*, "Hemolytic and antimicrobial activities of the twenty-four individual omission analogs of melittin," *Biochemistry*, vol. 30, pp. 4671-4678 (1991); Andreu *et al.*, "Shortened cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity," *Febs Letters*, vol. 296, pp. 190-194 (1992); Macias *et al.*, "Bactericidal activity of magainin 2: use of lipopolysaccharide mutants," *Can. J. Microbiol.*, vol. 36, pp. 582-584 (1990); Rana *et al.*, "Interactions between magainin-2 and *Salmonella typhimurium* outer membranes: effect of Lipopolysaccharide structure," *Biochemistry*, vol. 30, pp. 5858-5866 (1991); Diamond *et al.*, "Airway epithelial cells are the site of expression of a mammalian antimicrobial peptide gene," *Proc. Natl. Acad. Sci. USA*, vol. 90, pp. 4596 *ff* (1993); Selsted *et al.*, "Purification, primary structures and antibacterial activities of β-defensins, a new family of antimicrobial peptides from bovine neutrophils," *J. Biol. Chem.*, vol. 268, pp. 6641 *ff* (1993); Tang *et al.*, "Characterization of the disulfide motif in BNBD-12, an antimicrobial β-defensin peptide from bovine neutrophils," *J. Biol. Chem.*, vol. 268, pp. 6649 *ff* (1993); Lehrer *et al.*, *Blood*, vol. 76, pp. 2169-2181 (1990); Ganz *et al.*, *Sem. Resp. Infect. I.*, pp. 107-117 (1986); Kagan *et al.*, *Proc. Natl. Acad. Sci. USA*, vol. 87, pp. 210-214 (1990); Wade *et al.*, *Proc. Natl. Acad. Sci. USA*, vol. 87, pp. 4761-4765 (1990); Romeo *et al.*, *J. Biol. Chem.*, vol. 263, pp. 9573-9575 (1988); Jaynes *et al.*, "Therapeutic Antimicrobial Polypeptides, Their Use and Methods for Preparation," WO 89/00199 (1989); Jaynes, "Lytic Peptides, Use for Growth, Infection and Cancer," WO 90/12866 (1990); Berkowitz, "Prophylaxis and Treatment of Adverse Oral Conditions with Biologically Active Peptides," WO 93/01723 (1993).

Families of naturally-occurring lytic peptides include the cecropins, the defensins, the sarcotoxins, the melittins, and the magainins. Boman and coworkers in Sweden performed the original work on the humoral defense system of *Hyalophora cecropia*, the giant silk moth, to protect itself from bacterial infection. See Hultmark *et al.*, "Insect immunity. Purification of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*," *Eur. J. Biochem.*, vol. 106, pp. 7-16 (1980); and Hultmark *et al.*, "Insect immunity. Isolation and structure of cecropin D. and four minor antibacterial components from *cecropia* pupae," *Eur. J. Biochem.*, vol. 127, pp. 207-217 (1982).

Infection in *H. cecropia* induces the synthesis of specialized proteins capable of disrupting bacterial cell membranes, resulting in lysis and cell death. Among these specialized proteins are those known collectively as cecropins. The principal cecropins -- cecropin A, cecropin B, and cecropin D -- are small, highly homologous, basic peptides. In collaboration with Merrifield, Boman's group showed that the amino-terminal half of the various cecropins

contains a sequence that will form an amphipathic alpha-helix. Andrequ *et al.*, "N-terminal analogues of cecropin A: synthesis, antibacterial activity, and conformational properties," *Biochem.*, vol. 24, pp. 1683-1688 (1985). The carboxy-terminal half of the peptide comprises a hydrophobic tail. See also Boman *et al.*, "Cell-free immunity in Cecropia," *Eur. J. Biochem.*, vol. 201, pp. 23-31 (1991).

A cecropin-like peptide has been isolated from porcine intestine. Lee *et al.*, "Antibacterial peptides from pig intestine: isolation of a mammalian cecropin," *Proc. Natl. Acad. Sci. USA*, vol. 86, pp. 9159-9162 (1989).

Cecropin peptides have been observed to kill a number of animal pathogens other than bacteria. See Jaynes *et al.*, "In Vitro Cytocidal Effect of Novel Lytic Peptides on *Plasmodium falciparum* and *Trypanosoma cruzi*," FASEB, 2878-2883 (1988); Arrowood *et al.*, "Hemolytic properties of lytic peptides active against the sporozoites of *Cryptosporidium parvum*," *J. Protozool.*, vol. 38, No. 6, pp. 161S-163S (1991); and Arrowood *et al.*, "In vitro activities of lytic peptides against the sporozoites of *Cryptosporidium parvum*," *Antimicrob. Agents Chemother.*, vol. 35, pp. 224-227 (1991). However, normal mammalian cells do not appear to be adversely affected by cecropins, even at high concentrations. See Jaynes *et al.*, "*In vitro* effect of lytic peptides on normal and transformed mammalian cell lines," *Peptide Research*, vol. 2, No. 2, pp. 1-5 (1989); and Reed *et al.*, "Enhanced in vitro growth of murine fibroblast cells and preimplantation embryos cultured in medium supplemented with an amphipathic peptide," *Mol. Reprod. Devel.*, vol. 31, No. 2, pp. 106-113 (1992).

Defensins, originally found in mammals, are small peptides containing six to eight cysteine residues. Ganz *et al.*, "Defensins natural peptide antibiotics of human neutrophils," *J. Clin. Invest.*, vol. 76, pp. 1427-1435 (1985). Extracts from normal human neutrophils contain three defensin peptides: human neutrophil peptides HNP-1, HNP-2, and HNP-3. Defensin peptides have also been described in insects and higher plants. Dimarcq *et al.*, "Insect immunity: expression of the two major inducible antibacterial peptides, defensin and diptericin, in *Phormia terranvae*," *EMBO J.*, vol. 9, pp. 2507-2515 (1990); Fisher *et al.*, *Proc. Natl. Acad. Sci. USA*, vol. 84, pp. 3628-3632 (1987).

Slightly larger peptides called sarcotoxins have been purified from the fleshfly *Sarcophaga peregrina*. Okada *et al.*, "Primary structure of sarcotoxin I, an antibacterial protein induced in the hemolymph of *Sarcophaga peregrina* (flesh fly) larvae," *J. Biol. Chem.*, vol. 260, pp. 7174-7177 (1985). Although highly divergent from the cecropins and defensins, the sarcotoxins presumably have a similar antibiotic function.

Other lytic peptides have been found in amphibians. Gibson and collaborators isolated two peptides from the African clawed frog, *Xenopus laevis*, peptides which they named PGS

and $Gly^{10}Lys^{22}PGS$. Gibson *et* al., "Novel peptide fragments originating from PGL_a and the caervlein and xenopsin precursors from *Xenopus laevis*," *J. Biol. Chem.*, vol. 261, pp. 5341-5349 (1986); and Givannini *et al.*, "Biosynthesis and degradation of peptides derived from *Xenopus laevis* prohormones," *Biochem. J.*, vol. 243, pp. 113-120 (1987). Zasloff showed that the *Xenopus*-derived peptides have antimicrobial activity, and renamed them magainins. Zasloff, "Magainins, a class or antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial DNA sequence of a precursor," *Proc. Natl. Acad. Sci. USA*, vol. 84, pp. 3628-3632 (1987).

Synthesis of nonhomologous analogs of different classes of lytic peptides has been reported to reveal that a positively charged, amphipathic sequence containing at least 20 amino acids appeared to be a requirement for lytic activity in some classes of peptides. Shiba *et al.*, "Structure-activity relationship of Lepidopteran, a self-defense peptide of *Bombyx more*," *Tetrahedron*, vol. 44, No. 3, pp.787-803 (1988). Other work has shown that smaller peptides can also be lytic. See McLaughlin *et al.*, cited below.

Cecropins have been shown to target pathogens or compromised cells selectively, without affecting normal host cells. The synthetic lytic peptide known as S-1 (or Shiva 1) has been shown to destroy intracellular *Brucella abortus*-, *Trypanosoma cruzi*-, *Cryptosporidium parvum*-, and infectious bovine herpes virus I (IBR)-infected host cells, with little or no toxic effects on noninfected mammalian cells. See Jaynes *et al.*, "*In vitro* effect of lytic peptides on normal and transformed mammalian cell lines," *Peptide Research*, vol. 2, No. 2, pp. 1-5 (1989); Wood *et al.*, "Toxicity of a Novel Antimicrobial Agent to Cattle and Hamster cells *In vitro*," Proc. Ann. Amer. Soc. Anim. Sci., Utah State University, Logan, *UT. J. Anim. Sci. (Suppl. 1)*, vol. 65, p. 380 (1987); Arrowood *et al.*, "Hemolytic properties of lytic peptides active against the sporozoites of *Cryptosporidium parvum*," *J. Protozool.*, vol. 38, No. 6, pp. 161S-163S (1991); Arrowood *et al.*, "In vitro activities of lytic peptides against the sporozoites of *Cryptosporidium parvum*," *Antimicrob. Agents Chemother.*, vol. 35, pp. 224-227 (1991); and Reed *et al.*, "Enhanced in vitro growth of murine fibroblast cells and preimplantation embryos cultured in medium supplemented with an amphipathic peptide," *Mol. Reprod. Devel.*, vol. 31, No. 2, pp. 106-113 (1992).

Morvan *et al.*, "In vitro activity of the antimicrobial peptide magainin 1 against *Bonamia ostreae*, the intrahemocytic parasite of the flat oyster *Ostrea edulis*," *Mol. Mar. Biol.*, vol. 3, pp. 327-333 (1994) reports the *in vitro* use of a magainin to selectively reduce the viability of the parasite *Bonamia ostreae* at doses that did not affect cells of the flat oyster *Ostrea edulis*.